A comparative study of chitin synthase activity in two *Mortierella* species

by

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Abstract

An *in vitro* investigation of some important factors controlling the activity of chitin synthase in cell-free extracts of two *Mortierella* species has been carried out. Mixed membrane fractions from mycelial homogenates of *Mortierella candelabrum* and *Mortierella pusilla* were found to catalyse the transfer of N-acetylglucosamine from UDP-N-acetylglucosamine into an insoluble product characterized as chitin by its insolubility in weak acid and alkali, and the release of glucosamine and diacetylchitobiose on hydrolysis with a strong acid and chitinase, respectively. Apparent K_m values for UDP-GlcNAc were 1.8 mM and 2.0 mM for *M. pusilla* and *M. candelabrum*, respectively. Polyoxin D was found to be a very potent competitive inhibitor with values of the constant of inhibition, K_i, for both species about three orders of magnitude lower than the K_m for UDP-GlcNAc. A divalent cation, Mg^{+2}, Mn^{+2} or Co^{+2}, was required for activity. N-acetylglucosamine, the monomer of chitin, stimulated the activity of the enzyme. The crude enzyme preparation of *M. candelabrum*, unlike that of *M. pusilla*, showed an absolute requirement for both Mg^{+2} and N-acetyl-glucosamine. Large differences in response to exogenous proteases were noted in the ratio of active to inactive chitin synthase of the two species. A fifteen fold or greater increase was obtained after treatment with acid protease (from *Aspergillus saitoi*) as compared to a two- to four-fold activation of the *M. pusilla* membrane preparation treated similarly.

During storage at 4°C over 48 hours, an endogenous activation of chitin synthase of M. pusilla was achieved, comparable to that obtained by exogenous protease treatment. The high speed supernatant of both species inhibited the chitin synthase activity of the mixed membrane fractions. The inhibitor of M. pusilla was effective against the pre-activated enzyme whereas that of M. candelabrum inhibited the activated enzyme. Several possibilities are discussed as to the role of the different factors regulating the enzyme activity. The suggestion is made from the properties of chitin synthase in the two species that in vivo a delicate balance exists between the activation and inactivation of the enzyme which is responsible for the pattern of wall growth of each fungus.

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DEDICATION

I wish to dedicate this manuscript to my wife, Janet Adjimani, and my parents for their constant encouragement during the entire period of the study.

ABBREVIATIONS USED IN TEXT

Mc	*Mortierella candelabrum*
Mp	*Mortierella pusilla*
UDP-GlcNAc	Uridine diphosphate-N-acetyl-D-glucosamine
GlcNAc	N-acetyl-D-glucosamine
UMP	Uridine-5'-monophosphate
CWF	cell wall fraction
MMF	Mixed membrane fraction
SF	Supernatant (cytosolic) fraction

INTRODUCTION

The problems connected with the process of cell wall formation in fungi are being currently investigated from different points of view. Among the most frequently studied questions are: the molecular mechanisms of biosynthesis of individual cell wall components, the regulatory aspects at different levels of synthesis and cell wall formation in relation to morphological development of cells. Accordingly, different experimental systems have been used, ranging in degree of complexity from isolated enzymes or enzyme systems through subcellular fractions and protoplasts to intact cells. Studies on the mechanism of biosynthesis of cell surface structures using fungi as models for simple eukaryotic cells has become increasingly important. The reason for these studies is that they could not only explain the mechanism of biosynthesis of different cell wall components, but also contribute to a better understanding of various surface related biological phenomena such as cell-cell interactions, immune response, morphogenesis and drug resistance.

Characterization of cell walls of mucoraceous fungi, including the Mortierella species, acting as hosts to mycoparasites is a subject of investigation in our laboratory. Besides providing sites of attachment for the parasite, the host cell surface offers the first line of defense against the parasite. It has been suggested by Manocha (unpublished report) that an investigation of surface ultrastructure and chemistry might prove

rewarding. Chitin is a major component of cell walls of the fungal class to which the Mortierella species belong (Bartnicki-Garcia, 1968). It is found in most fungi in the form of microfibrils along with fibrilar (1-3)-β-D-glucan (Kreger and Kopecka, 1975; van der Valk et al., 1977). Surprisingly, very little is found in the literature concerning the molecular mechanism of glucan biosynthesis in fungi. In spite of considerable effort, the numerous attempts to demonstrate in vitro biosynthesis of glucan from different nucleoside diphosphate glucose precursors in cell-free systems have failed (Cabib, 1975). The lack of success in most of these experiments indicate that the enzyme system catalysing the synthesis of wall glucan in fungi is more delicate and more sensitive to damage than are the other fungal polysaccharide synthases. Contrary to the situation with the enzyme system involved in glucan synthesis, chitin synthase, the enzyme which catalyses the last reaction in the pathway of chitin synthesis, is the best characterized enzyme involved in the biosynthesis of hyphal walls. Previous studies on this enzyme in yeasts and other fungi have indicated that enzymatic activity is modulated by a variety of controls including the rate of synthesis, proenzyme activation and allosteric control (Cabib and Farkas, 1971; McMurrough and Bartnicki-Garcia, 1971). The discovery of an activating factor and its specific inhibitor led to an assumption that they might be components of the system regulating yeast chitin synthase in vivo (Cabib and Keller, 1971). With regard to the crucial morphogenetic role of chitin in fungal cell walls, the interconversion between the zymogen and

the active enzyme might represent a key mechanism for the regulation of chitin synthesis.

In the present study, an *in vitro* characterization of chitin synthase in cell-free extracts of *Mortierella candelabrum* and *Mortierella pusilla* has been investigated. This has been considered a useful experimental system to determine which factors are most important in controlling the activity of the enzyme in the two species. Electron micrographs prepared from thin sections of young intact hyphae of these species revealed differences in thickness of their electron opaque cell walls (Manocha, unpublished results). The wall of *M. pusilla* is thicker and shows distinct layers, whereas that of *M. candelabrum* is thin with no indication of distinct layers. Furthermore, these two species differ in their cell surface interactions with the mycoparasite *Piptocephalis virginiana*. Whereas the parasite develops a contact and an appressorium at the surface of the compatible host, *M. pusilla*, it fails to do so in the non-host, *M. candelabrum*.

LITERATURE REVIEW

Chitin is a substance of considerable biological importance. It consists of (1-4)-β-D-glucose chains with every C(2) substituted by an acetylamino group (Aspinal, 1970). It is found as a major component of fungal cell walls and occurs in more than one crystal form. The α-chitin, which is more common than β-chitin, is the form encountered in fungi. Chitin is almost always found as crystalline microfibrils usually embedded in a matrix constituted of other polysaccharides. For example, in Saccharomyces cerevisiae (Kreger and Kopecka, 1975), chitin is found in the form of microfibrils along with fibrilar (1-3)-β-D-glucan. The latter is also found in other species as a matrix polysaccharide embedding the chitin microfibrils as in Schizophyllum commune (van der Valk et al., 1977). In all systems examined in detail, chitin microfibrils lie in the innermost layer of the fungal wall adjacent to the plasmalemma and their mechanical strength suggests that they play a major role in the determination of the shape of the fungal cell, whilst withstanding the turgor pressure exerted by the cytoplasm (Hunsley and Burnett, 1970).

Chitin was first discovered in fungi by Braconnot (1811) and since then much has been published on enzymes involved in its degradation. However, the mechanism for controlling the rate of synthesis remains obscure. The first evidence for an in vitro chitin synthesis was obtained by Glaser and Brown (1957) who demonstrated in Neurospora crassa

that uridine diphosphate-N-acetyl-D-glucosamine (UDP-GlcNAc) is the precursor for the synthesis of a polymer recognized as chitin by its insolubility in water, alkali and weak acids and by its digestibility with strong acids and chitinase. The most convincing evidence for its identity with chitin of the cell wall comes from the demonstration that the product can be visualized as crystalline microfibrils (Ruiz-Herrera and Bartnicki-Garcia, 1974). Subsequent workers have repeatedly confirmed the ability of UDP-GlcNAc to serve as a precursor for chitin synthesis in cell-free extracts from a wide taxonomic range of chitin-containing fungi (Jaworski, Wang and Carpenter, 1965; Camargo et al., 1967; Keller and Cabib, 1971; McMurrough et al., 1971; Jan, 1974; Peberdy and Moore, 1975; Gooday and de Rousset-Hall, 1975; Lopez-Romero and Ruiz-Herrera, 1976; Braun and Calderone, 1978; Hanseler et al., 1983) and its properties appear to be very similar from all these sources. The general equation for the reaction is:

$$\text{UDP-GlcNAc} + [\beta\text{-}(1,4)\text{-GlcNAc}]_n \xrightarrow{Mg^{+2}} [\beta\text{-}(1,4)\text{-GlcNAc}]_{n+1} + \text{UDP}$$

A single enzyme UDP-2-acetamido-2-deoxy-D-glucose:chitin 4-β-acetamido deoxyglucosyltransferase (EC 2.4.1.16) known under the trivial name chitin synthase seems to be involved in the reaction. In common with other enzymes with a nucleotide sugar as substrate, chitin synthase preparations have a requirement for divalent cations, particularly Mg^{+2} as co-substrate. Results obtained with various enzyme preparations

indicate that chitin synthase is an allosteric enzyme having more than one binding site per molecule (de Rousset-Hall and Gooday, 1975; McMurrough and Bartnicki-Garcia, 1971). The transfer of GlcNAc from UDP-DlcNAc to a primer molecule seems to proceed in a single step, and there is, so far, no evidence about participation of "lipid intermediate" in the enzymatic reaction (Endo and Misato, 1969; McMurrough et al., 1971) or for a primer requirement (Gooday and de Rousset-Hall, 1975; Duran and Cabib, 1978) and it appears that the enzyme is fairly autonomous and can carry out the synthesis of the polysaccharide by a direct transfer of N-acetylglucosamine in the cell. This mechanism may, however, be complicated by the fact that the substrate UDP-GlcNAc is intracellular, whereas the product is laid down outside the plasma membrane. The process of chitin extrusion may require the participation of some other component (Duran and Cabib, 1978). No such constraints operate in vitro, and this could account for the observation (Gooday and de Rousset-Hall, 1975) that the purified preparation of the enzyme is capable of synthesizing long chains of chitin. An endogenous primer may, of course, be present in the preparation, but the properties of chitin and the manipulations undergone by the enzyme pose severe limitations on its possible nature. A short oligosaccharide would be soluble, but it would have to be strongly, perhaps covalently bound to the enzyme in order to accompany it during column chromatography (Duran and Cabib, 1978). The observation that GlcNAc is an allosteric effector has been considered one of purely laboratory significance as this sugar does not occur in measurable quantities in fungal cells. However, the one place that it will occur is

at the site of enzyme lysis of chitin by a concerted action of chitinase and diacetylchitobiase. Thus, it could activate chitin at those sites where lysis and synthesis must go hand in hand, namely at the point of hyphal hyphal branch formation and almost certainly at the growing apex (Gooday and de Rousset-Hall, 1975). The dimer, diacetylchitobiose and higher chitin oligomers, both products of chitinase activity, also activate in vitro chitin biosynthesis, although usually not as powerfully as the monomer. It has been suggested that these molecules could act directly as primers for the enzyme activity, but the evidence has not been conclusive and in most enzyme preparations this has been shown not to occur to any significant extent (McMurrough et al., 1971; Peberdy and Moore, 1975). In vivo there is presumably usually no shortage of partly formed or recently lysed chitin chains to act as acceptors (de Rousset-Hall and Gooday, 1975).

The enzyme product, uridine diphosphate (UDP), is an inhibitor of chitin synthase, having an apparent K_i value of 0.6 mmole L^{-1} with the enzyme of Coprinus cinereus. Enzyme preparations commonly contain a nucleoside diphosphatase that acts on UDP and converts it to uridine monophosphate (UMP) which is not nearly so inhibitory (Gooday and de Rousset-Hall, 1975). One of the most powerful inhibitors of fungal chitin synthase is the pyrimidine antibiotic, polyoxin D (Endo and Misato, 1969). The inhibition of polyoxin D is competitive with regard to the substrate UDP-GlcNAc, the inhibitor constant, K_i, being two to three orders of magnitude lower than the Michaelis constant, K_m, for the substrate. A mechanism has been suggested whereby polyoxin D, owing to its structural similarity with UDP-GlcNAc, competes for the active site of enzyme (Hori et al., 1974).

Fungal chitin synthase is normally prepared as a particulate enzyme associated with cellular membranes. Evidence from fluorescence and autoradiographic experiments confirmed the presence of chitin synthase on the plasma membrane (Duran et al., 1975). Other studies with cell-free extracts from various fungi have indicated that the highest specific activity of chitin synthase is present in a membrane fraction vaguely termed "Microsomal" (Gooday, 1971; Lopez-Romero and Ruiz-Herrera, 1976). Similarly, isopycnic preparations from Candida albicans (Braun and Calderone, 1978) point out that the plasma membrane is the principal site of chitin biosynthesis. Nevertheless, there have been repeated claims (McMurrough et al., 1971; Peberdy and Moore, 1975) that the bulk of chitin synthase is located directly in the cell wall. One of the factors negatively influencing the isolation of membrane fractions might be that mechanical disintegrations of the cells before enzyme extraction as well as bursting of protoplasts by osmotic shock which could cause fragmentation of cellular membranes and their vesiculation (Dube et al., 1973). Using concanavalin A to preserve the integrity of the plasma membrane, Duran et al., (1975) were able to show that yeast chitin synthase is located exclusively in the plasma membrane. The fact that fungal protoplasts can be used effectively for preparation of the enzyme is also against the claim for a predominant location of chitin synthase in cell walls. The membrane bound chitin synthase can be liberated by butanol extraction (Glaser and Brown, 1957) by digitonin treatment (Gooday, 1971) or by incubation of the particulate enzyme with the substrate and activator

(Ruiz-Herrera and Bartnicki-Garcia, 1974). Whereas butanol and digitonin destroy the basic membrane structure, the solubilizing effect of UDP-GlcNAc together with GlcNAc has been difficult to explain. Ultrastructural studies revealed a rather complicated structure of chitin synthase particles (microvesicular chitosomes) (Bracker et al., 1976). Freshly isolated chitosomes appear on ultrathin sections as clusters of protein granules bound within a membranous shell. Within such chitosomes, fine fibrils, 1-2 nm in diameter and possessing the insolubility properties of chitin can be recognized. More recently, functional chitosomes have been isolated from a series of different genera of fungi, including yeasts (Bartnicki-Garcia et al., 1978). Although chitosomes are fully capable of synthesizing chitin microfibrils in vitro under appropriate conditions, their existence in vivo has not been fully confirmed. With regard to their relatively large dimensions (40-70 nm in diameter), they cannot be considered as integral components of the plasma membrane or another cellular membrane (average membrane thickness is 8-9 nm) (Ruiz-Herrera and Bartnicki-Garcia, 1976). Chitosomes could possibly represent containers of chitin synthase conveying the enzyme from the site of its synthesis to its destination at the cell surface, or eventually they could be of artifactual nature. The latter nature of these minor organelles is indicated by the fact that molecules of chitin synthase solubilized from the cell walls of Mucor rouxii by digitonin treatment associate with one another to form vesiculoid structures morphologically and functionally resembling chitosomes (Bartnicki-Garcia et al., 1977).

Perhaps the most remarkable property of fungal chitin synthase is that it exists in cells largely in an inactive or zymogenic state. The inactive enzyme can be converted to the active form by limited proteolysis. The phenomenon of proteolytic activation was first discovered with yeast chitin synthase (Cabib and Keller, 1971) and later confirmed with preparations from other sources (McMurrough and Bartnicki-Garcia, 1973; Gooday and de Rousset-Hall, 1975; Lopez-Romero and Ruiz-Herrera, 1976; Ryder and Peberdy, 1977; van Laere and Carlier, 1978). The zymogenic character was also detected in solubilized preparations of the enzyme (Ruiz-Herrera and Bartnicki-Garcia, 1976). Crude preparations of yeast chitin synthase obtained from protoplast lysate underwent a slow but significant increase in specific activity upon standing or storage at low temperatures, indicating that they might contain some intrinsic factor capable of activating chitin synthase (Cabib and Farkas, 1971). Activation of the yeast enzyme could also be achieved by incubation with an extract from the same yeast, which contains an "activating factor". Mild sonication of the pellets from the protoplast lysates led to the solubilization of the so-called activating factor whereby chitin synthase remained insoluble. The chitin synthase freed from the activating factor had only negligible activity unless it was pre-incubated with the activating factor (Cabib and Farkas, 1971). Subsequent studies have shown that the activating factor is a protease located in some kind of intracytoplasmic vesicle, inseparable from the vacuolar fraction (Cabib et al., 1973; Cabib and Ulane, 1973). This finding provided a possible

explanation for the initiation of chitin synthesis at the budding site, an event that is precisely determined with respect to spatial location and temporal position in the cell cycle. It seemed probable that chitin synthesis could be triggered by contact between chitin synthase zymogen and activating factor (Cabib and Farkas, 1971), although it is not clear how the localization and time of this event can be achieved. Many proteases from other sources, for example, trypsin, subtilism, rennilase and acid protease are also capable of activating chitin synthase. However, the activating effect of the individual proteases is not the same in all cases (Lopez-Romero and Bartnicki-Garcia, 1978). The zymogen enzyme from various fungi responded differently to neutral or acid proteases (Bartnicki-Garcia et al., 1978). The enzyme from M. rouxii was activated to higher levels by rennilase than by trypsin. Conversely the enzyme of Neurospora crassa and Agaricus bisporus responded better to trypsin than to rennilase. There was also a considerable variation in the ratio of zymogen to active enzyme in the purified chitosome pool, but these differences were probably the result of uncontrolled activation by endogenous proteases released during cell breakage (Bartnicki-Garcia, et al., 1978). Cabib and Farkas (1971) found that in crude particulate preparations from Saccharomyces species, chitin synthase was essentially in an inactive form. The supernatant fraction obtained after lysis of yeast protoplasts has been found to contain a heat-stable protein capable of selective binding to the activating factor, thus rendering it ineffective (Cabib and Farkas, 1971). The inhibitor of the activating factor has been purified

to homogeneity and found to be a low molecular weight peptide (molecular weight, 8,500) which forms a tight complex with the activating factor in a stoichiometric ratio of one to one (Ulane and Cabib, 1974). Lopez-Romero et al. (1978), isolated from extracts of M. rouxii another protein capable of inhibiting the in vitro synthesis of chitin. In contrast to the inhibitor from Saccharomyces cerevisiae, the isolated substance of M. rouxii did not interfere with proteolytic activation of the zymogen by the activating factor, but it directly inhibited the pre-activated chitin synthase. A working hypothesis for the mechanism of regulation of chitin synthase activity in yeasts has been proposed by Cabib et al., 1973, based on the properties of the individual components of the system catalysing the formation of chitin. According to this scheme, chitin synthase zymogen is uniformly distributed on the plasma membrane and is activated at specific sites (such as those of bud formation) by the impact of vesicles containing the activating factor. The inhibitor functions as a safety valve that might be released into the cytoplasm and thus prevent activation of zymogen elsewhere on the plasma membrane. Although plausible, the proposed scheme for regulation of chitin synthesis in yeast remains still in the category of working hypothesis.

MATERIALS AND METHODS

(i) Organisms and growth conditions

Cultures of Mortierella candelabrum, v. Teigh and Le Monn, and Mortierella pusilla, Oudemans, were routinely maintained at 25° ± 1°C on solid medium consisting of malt extract, 20 g; yeast extract, 5 g; and agar 20 g, in a litre of distilled water. Spores were harvested from 7 to 10 days old cultures and the spore suspensions in distilled water were adjusted to a final concentration of 10^9 mL^{-1} and used to inoculate 250 mL of malt-yeast extract medium contained in 500 mL flasks. The cultures were incubated for 24 h at 25°C on a G24 Environmental Incubator Shaker (New Brunswick Scientific Instruments) at 150 rev. min^{-1}.

(ii) Preparation of enzyme

The mycelium from the above growth was harvested by filtration with suction through a Buchner funnel and trapped on a qualitative-grade (Whatman No. 4) filter paper. The mycleium was then washed several times with distilled water and once in cold 25 mM KH_2PO_4/NaOH buffer, pH 7.0. Subsequent operations were performed at 0 to 4°C.

About 5 g fresh weight of mycelium suspended in 25 mL of homogenizing buffer (25 mM KH_2PO_4/NaOH buffer, pH 7.0) was filled into a 50 mL glass container and glass beads (0.45-0.50 mm in diameter) added in a ratio of 3:1 parts by weight. The mycelium was disrupted for 40 sec at 4000 rpm in

a Braun MSK homogenizer cooled with liquid carbon dioxide. The homogenized mycelium was centrifuged at 1000 g for 5 min to remove unbroken hyphae and cell wall fraction (CWF). This fraction was washed five times with homogenizing buffer to remove contaminating membranous material and resuspended in 10 mL of the same buffer. The supernatant was centrifuged at 100,000 g for 40 min using an IEC/B-60 ultracentrifuge. The pellet was washed once in the homogenizing buffer and resuspended in 15 mL of the same buffer, using a Potter-Elvehjem (pestle) homogenizer, in an ice-cooled thick-walled test tube. This suspension was designated the "mixed membrane fraction" (MMF). The supernatant from the high speed centrifugation was designated "supernatant fraction" (SF). The subcellular preparations, (CWF, MMF and SF) were used immediately for chitin synthase assay without further treatment unless otherwise stated.

(iii) Chitin Synthase Assay

The assay procedure was similar to that described by Ruiz-Hererra and Bartnicki-Garcia (1974). The standard incubation mixture contained in a total volume of 180 µL, enzyme preparation (80 µL) with up to about 0.5 mg protein, UDP-[^{14}C]GlcNAc (0.5 mM, 20,000 dpm), GlcNAc (50 mM), $MgCl_2$ (20 mM) and 25 mM KH_2PO_4/NaOH buffer, pH 6.2. Where indicated enzyme preparations were pre-incubated with different concentrations of various exogenous proteases; a crystalline acid protease (from Aspergillus saitoi), trypsin (from bovine pancreas) and a neutral protease (from Bacillus amyloliquefaciens) to activate any zymogenic chitin synthase present in the subcellular fractions.

The reaction was started by adding 80 µL of activated or non-activated enzyme preparation to 100 µL of the reaction mixture in 10 mL test tubes. The assay mixture was incubated at 30°C for 5 min and the reaction terminated by adding 25 µL of glacial acetic acid. The content of each assay tube was filtered under vacuum onto a glass-fibre disc (Whatman GF/A, diameter 2.4 cm) held in a millipore filtration unit. The residue was washed with about 50 mL of 1 M acetic acid: 95% ethanol mixture (4:1, v/v) and dried at 70°C. The radioactive chitin formed during the reaction was retained on the filter fibre disc, possibly bound to the precipated protein and the soluble (unreacted) assay components were washed off. Further radioactive material could not be removed from the disc by repeated washing. The dried discs were placed in 15 mL scintillation fluid (Aqueous counting scintillant, Amersham/Searle Corp., Arlington, Illinois) and radioactivity counted in a Searle Delta 300 liquid scintillation spectrometer. Sample counting efficiency was determined by measuring the radioactivity in the evaporated filtrate from the incubation mixture. From the counts in the filtrate and the loss in count rate of the sample on the fibre discs, the sample counting efficiency was calculated from the total amount of radioactivity in the assay mixture at the start of the reaction. Chitin synthase activity was determined from the percentage incorporation of radioactivity into insoluble product, calculated from the equation in Appendix 4, and expressed as nmole GlcNAc incorporated min^{-1}. Specific activity was calculated as per mg of protein. Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Non-enzyme catalysed reactions were tested by control experiments in which heat-inactivated enzyme was added to the assay mixture. All assays were performed in duplicate unless stated otherwise.

(iv) Characterization of the reaction product

Chitin synthase was characterized by a method similar to that described by McMurrough et al. (1971). Standard reaction mixtures were scaled up to 2 mL and incubated for 5 min. The reaction was terminated by the addition of 100 µL of glacial acetic acid. Samples (0.2 mL portions) of the incubated mixture were either spotted on strips of Whatman No. 3MM paper and irrigated for 24 hours by descending flow using 95% ethanol-1 M ammonium acetate, pH 3.8 (7:3 v/v) or filtered through standard laboratory glass microfibre discs (GF/A). The radioactivity which remained at the origin of the chromatograms or on the discs was taken as a measure of chitin synthesis. Accordingly the dried discs or excised chromatogram origins were placed in vials containing 15 mL of scintillation fluid and counted for radioactivity.

Further identification of the biosynthesized product as chitin was carried out by dialysing 1 mL of the incubated mixture from the above assay against three changes of distilled water (500 mL) to remove unreacted substrate. The non-dialysable material was centrifuged at 2000 g for 10 min to yield a radioactive sediment and a clear radioactive supernatant. Portions from both fractions were counted to give the initial insoluble and soluble radioactivity. The radioactive sediment was suspended in 5.0 mL of

distilled water. Samples (0.5 to 1.0 mL) of this suspension were used for the following treatments:

Acetic acid hydrolysis

The radioactive suspension (1 mL) was heated in 5 mL of 1 M acetic acid for 30 min at 100°C and centrifuged at 2000 g for 10 min. The supernatant was evaporated under reduced pressure on a rotavapor and taken up in 0.2 mL of 1 M acetic acid and chromatographed using a Whatman No. 1 paper and developed in 95% ethanol/1M ammonium acetate, pH 3.8 (7:3, v/v) mixture. A suspension of the sediment was also chromatographed and radioactivity estimated. Immobile radioactivity was determined from excised chromatogram origins by counting in 15 mL of aqueous counting scintillant.

Radioactive spots on the chromatograms were located by visualization using N-acetylglucosamine and glucosamine standards. The amino sugar was detected by spraying with 0.5% solution of ninhydrin in butanol and the N-acetylated amino sugar was revealed as a purple spot by p-dimethylaminobenzaldehyde (Partridge and Westall, 1948).

Alkaline hydrolysis

One mL of the radioactive suspension was heated in 5.0 mL of 1 M KOH, for 1 hour at 100°C and then centrifuged for 10 min at 2000 g. Samples (0.2 mL) of the sediment and the supernatant were counted for radioactivity by liquid scintillation counting. Samples were also chromatographed and developed as described above.

Hydrochloric acid hydrolysis

The non-dialysable radioactive suspension (1 mL) was hydrolysed in 5.0 mL of 6 M HCl at 120°C for 4 hours in vacuo. The hydrolysate was evaporated on a rotavapor and the residue taken up in 1 mL of water and centrifuged at 2000 g for 10 min to precipitate unhydrolysed material. Suspensions of the sediment were counted for radioactivity by liquid scintillation counting. Samples (0.2 mL) of the supernatant were also chromatographed and developed as above.

Chitinase digestion

A suspension (0.8 mL) of the non-dialysable radioactive product was incubated with chitinase (5 mg of protein) in 0.2 mL sodium acetate buffer pH 5.0 (0.2 M) for 8 hours at 30°C. The reaction was terminated by the addition of 100 µL of glacial acetic acid and then centrifuged at 2000 g for 10 min. The sediment and supernatant were counted for radioactivity. Samples from these fractions were chromatographed and developed in two solvent systems: Ethylacetate-pyridine-water (2:2:1) and methanol-pyridine-acetic acid-water (6:6:1:4). Radioactive spots were detected by comparing the R_f values with those obtained using authentic diacetyl-chitobiose and N-acetylglucosamine.

To ascertain the fate of the substrate (UDP-N-acetylglucosamine) remaining after incubation, the supernatant obtained after centrifugation of the standard incubated mixture was evaporated on the rotavapor and submitted to paper chromatography with 95% ethanol-1 M ammonium acetate

(7:3, v/v) mixture. UDP-GlcNAc in the supernatant was detected by counting excised chromatograms, at areas which migrated with the same R_f as authentic UDP-GlcNAc, for radioactivity.

(v) Inhibition of chitin synthase activity by high speed supernatant fraction

The supernatant (50 mL) from the high speed (100,000 g) centrigufation was lyophilized on a Labconco Freeze Dryer-5 (Fisher Scientific Instruments) and resuspended in 5 mL of homogenizing buffer. Portions (10 µL) of the suspended fraction were either added to 100 µL of the standard incubation mixture just before the start of the reaction or to the crude enzyme preparation (100 µL/mL of enzyme) for 10 min before being added to the incubation mixture.

(vi) Endogenous protease activity

(i) Measurement with hemoglobin as substrate

The method used in this assay is similar to that described by Anson (1939). In this procedure, hemoglobin is denatured with alkaline urea solution. Proteases hydrolyse compounds from the denatured protein which are soluble in trichloroacetic acid and whose tyrosine and tryptophan content can be determined by the method of Folin and Ciocalteu (1927). The incubation mixture contained: 5.0 mL hemoglobin (16.7 mg/mL), 1.0 mL of mixed membrane fraction (3.0-3.5 mg protein) or supernatant fraction (1.0-1.5 mg protein) prepared as described earlier. The substrate and enzyme solutions were mixed and incubated for exactly 10 min at 25°C. The

reaction was stopped by adding 10.0 mL 5% trichloroacetic acid. The reaction mixture was shaken vigorously and allowed to stand for 30 min at room temperature and centrifuged for 20 min at 4000 g. Blanks were prepared in which the hemoglobin was first precipitated with trichloroacetic acid before the enzyme preparation was added. A colour reaction was developed by pipetting into 50 mL flasks 5.0 mL of the supernatant from the 4000 g centrifugation and adding 10 mL of 0.5 M NaOH. With continuous shaking, 3.0 mL of phenol reagent prepared by diluting stock solutions three-fold with distilled water was added. Precipitates were centrifuged off at 4000 g for 5 min. The extinction of the solution was measured between 5 and 10 min after addition of the phenol reagent at 691 nm using a Bausch 20 Spectrometer. Endogenous protease activity was expressed as μmole of soluble tyrosine (mg protein)$^{-1}$ h^{-1}. The amount of tyrosine was estimated by means of a tyrosine standard curve.

(ii) Measurement with casein as substrate

The method employed in this assay is similar to that described for hemoglobin and is due to Kunitz (1947). During hydrolysis of casein by trypsin, products soluble in trichloroacetic acid are formed and the tyrosine and tryptophan content is determined by measurement of the extinction at 280 nm. The reaction mixture contained in a total volume of 2.0 mL, 1.0 mL of mixed membrane fraction or supernatant fraction, and 1.0 mL of casein solution (5 mg casein mL^{-1}). The substrate solution and enzyme preparation were incubated for exactly 20 min at 35°C. The reaction

was terminated by the addition of 3.0 mL of 5% trichloroacetic acid. The incubated mixture was mixed thoroughly and allowed to stand for 30 min at room temperature and then centrifuged for 20 min at 3000 g. Extinction of the supernatant was measured at 280 nm in a Turner Photospectrometer (Model 330). Standards were run using 1-25 µg of trypsin. Blanks were prepared in which the casein was first precipitated with trichloroacetic acid before the enzyme solution was added.

Protease activity was calculated as "trypsin unit", TU^{cas}, (Kunitz, 1947). One TU^{cas} unit is the amount of trypsin which under defined conditions (20 min incubation at 35°C, final volume of the incubation mixture 2.0 mL; after addition of trichloroacetic acid: 5 mL) liberates sufficient trichloroacetic acid-soluble hydrolysis products so that the extinction at 280 nm increases by 1.0 in 1 min. Specific endogeneous protease activity was expressed as TU^{cas} mg^{-1} protein.

(iii) Measurement with synthetic substrate (Nα-benzoyl-DL-arginine-p-nitroanilide, BAPA)

The method used is similar to that described by Erlanger et al. (1961). To 5 mL of substrate solution (10^{-3} M), prepared by dissolving 43.5 mg DL-BAPA in 1 mL of dimethylsulfoxide and bringing the solution up to 100 mL with 0.05 M Tris buffer, pH 8.2 containing 0.02 M $CaCl_2$, was added 1 mL of mixed membrane preparation or supernatant fraction. The reaction was incubated for 10 min and stopped by adding 1.0 mL of 30% acetic acid. The quantity of p-nitroaniline liberated was estimated spectrometrically at

410 nm in a Bausch and Lomb Spectronic 20 spectrometer. A control was run in which there was no enzyme preparation.

RESULTS

A comparison of the properties of chitin synthase of Mortierella candelabrum and Mortierella pusilla has been carried out using mixed membrane fractions (MMF) from the 100,000 g centrifugation as described in the "Materials and Methods". The assay method was rapid and reproducible, the standard deviation within triplicate assays was routinely less than 10% of the mean value. The method was compared with one involving paper chromatography of the reaction mixture (Peberdy and Moore, 1975) and similar results were obtained, confirming that the product was completely retained on the glass fibre discs. The substrate did not adhere to the filters and control assays using heat-inactivated enzyme, generally gave average counts less than 20% above the background. Sample counting efficiency was typically 60%.

Freshly prepared acid protease activated mixed membrane preparations had an average chitin synthase activity of 4.0 ± 0.5 and 2.0 ± 0.5 nmole GlcNAc incorporated min^{-1} $(mg\ protein)^{-1}$ for M. pusilla and M. candelabrum respectively. The enzyme activity in both species depended on the length of time the mycelium was homogenized. Maximum activity was achieved after 30 to 40 sec, further homogenization for 20 sec reduced the activity by about 40 to 50 %. Results show data of one representative experiment of three replicates.

Subcellular distribution of chitin synthase

Mycelium homogenate was prepared as described in the "Materials and Methods" and centrifuged for 5 min at 1000 g to collect the cell wall fraction (CWF). The supernatant was centrifuged for 40 min at 100,000 g to obtain the pellet (MMF) and a supernatant designated the cytosol fraction (SF). Chitin synthase activity in each fraction was determined in the presence or absence of acid protease (Aspergillus saitoi) at a final concentration of 1000 μg mL^{-1}. Different degrees of stimulation of the enzyme were obtained in the various fractions. From the results in Figure 1, it appears that in the presence of acid protease more than 80% of chitin synthase activity of both species was associated with the particulate mixed membrane fraction. In the absence of the protease, the cell wall and supernatant fractions of the two species exhibited negligible chitin synthase activity. The distribution studies revealed that the mixed membrane fractions of M. candelabrum and M. pusilla had significant proportions of the total chitin synthase activity.

Identification of the reaction product

Chromatography of the sediment from the non-dialysable incubated mixture suspended in water revealed that more than 90% of the initial radioactivity was retained at the origin. The percentage of radioactivity retained at the origin was expressed as a ratio of counts from the excised chromatogram origin to that retained on the glass-fibre discs. Accordingly

95% of the product retained on the glass fibre discs could be chitin, if immobility of the reaction product from the origin of the chromatograms, as has been shown to be the case (McMurrough et al., 1971), is to be used as a criterion for the identification of the reaction product as chitin.

Further characterization of the polymer formed from UDP-GlcNAc was carried out by subjecting the reaction product to enzyme, acid and alkali hydrolysis as described under "Materials and Methods". Chitinase digestion revealed that only 15% of the original radioactivity remained insoluble, the rest was shown to be mainly diacetylchitobiose by comparing the R_f values to those obtained using authentic diacetylchitobiose samples in the same solvent system. On hydrolysis with concentrated hydrochloric acid about 80% of the initial radioactivity was recovered in solution. Paper chromatography revealed glucosamine as the only component of the soluble radioactive fraction. The synthesized product was soluble in neither 1 M acetic acid nor 1 M KOH as more than 80% radioactivity was recovered in the insoluble fraction after centrifugation.

Paper chromatography of the supernatant obtained from the standard assay mixture, using 95% ethanol/1 M ammonium acetate mixture (7:3 v/v) revealed a single radioactive spot corresponding to UDP-GlcNAc. Thus, hydrolysis of the substrate by the enzyme preparation was not detectable.

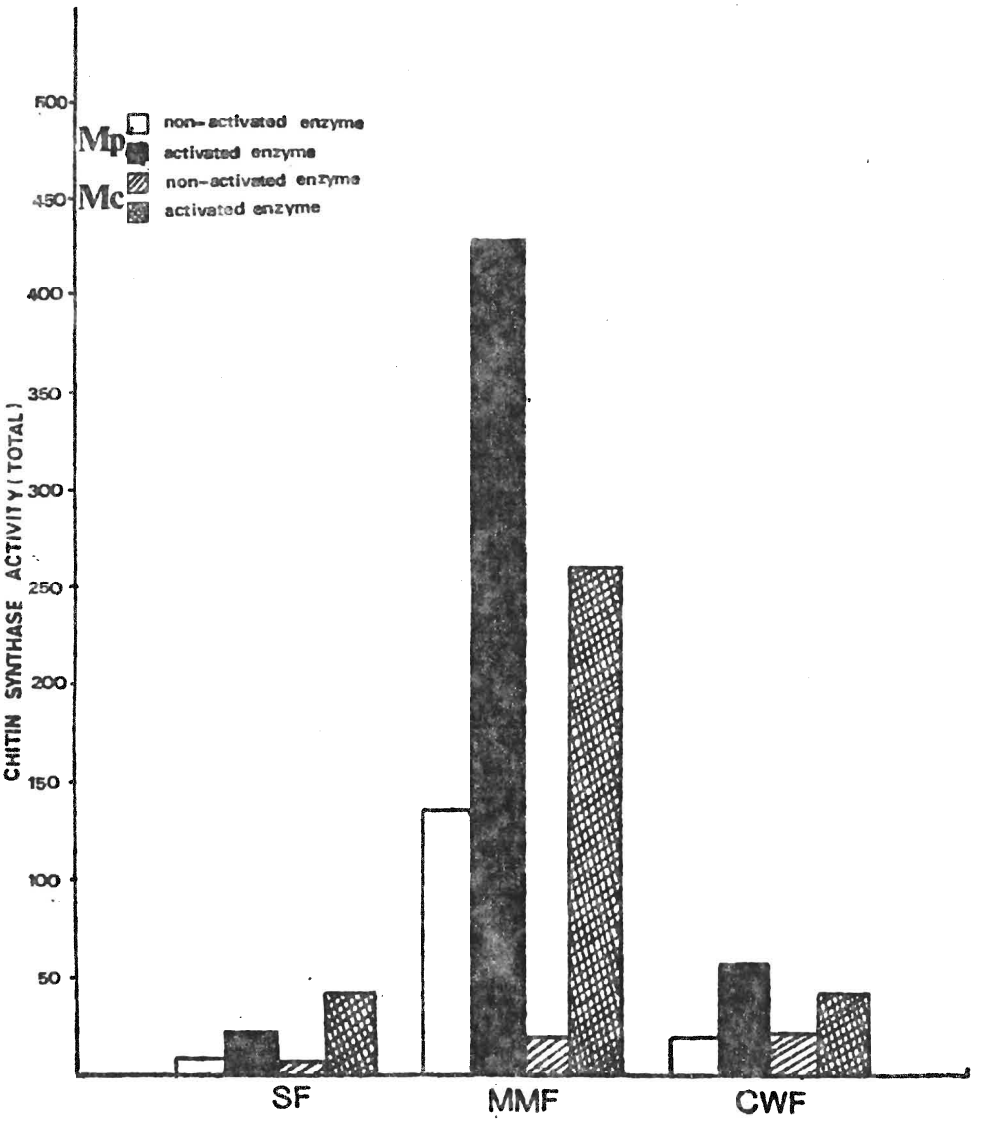
Figure 1. Distribution of chitin synthase in subcellular fractions of *M. candelabrum* and *M. pusilla*.

Chitin synthase activity was assayed in enzyme fractions (CWF, MMF and SF) prepared as described under "Materials and Methods". Enzyme activity was measured in both acid protease activated and non-activated preparations. Enzyme activation was achieved by pre-incubating the enzyme preparation with acid protease (1000 μg mL^{-1}) for 30 min. Total activity is expressed as nmole GlcNAc incorporated into chitin min^{-1} by each entire fraction. Protein content of each fraction was determined by Lowry's method; CWF (1.5-2.5 mg mL^{-1}); MMF (4-5 mg mL^{-1}); SF (2-2.5 mg mL^{-1}).

M. candelabrum (Mc)

M. pusilla (Mp)

Figure 1.


Mp
non-activated enzyme
activated enzyme
Mc
non-activated enzyme
activated enzyme
CHITIN SYNTHASE ACTIVITY (TOTAL)
500
450
400
350
300
250
200
150
100
50
SF
MMF
CWF

Properties and kinetics of chitin synthase of *M. candelabrum* and *M. pusilla*

Some properties of chitin synthase of the two species used in this study are compared with those of other fungi in Appendix 1.

(i) Effect of substrate and enzyme concentration

The incorporation of label into material insoluble in glacial acetic acid was linear during the first 5 min of incubation and then declined when approximately 40% and 20% of the substrate had been converted to chitin by the activated mixed membrane fraction of *M. pusilla* and *M. candelabrum*, respectively (Appendices 2a and 2b). The reaction rate was also a linear function of substrate (UDP-GlcNAc) concentration and remained constant up to 5 min for substrate concentrations above 0.2 mM UDP-GlcNAc (Appendices 2a and 2b). Enzyme activity declined sharply at substrate concentrations of 2 mM UDP-GlcNAc or below. The decrease in reaction rate after 5 min might have been due to depletion of substrate or accumulation of UDP, an inhibitory product of the reaction. Hydrolysis of the substrate by the enzyme preparation was not detected by paper chromatography of the supernatant from the incubated assay mixture and could not be the cause for the decrease in rate observed.

Chitin synthase activity also varied linearly with the concentration of enzyme in the reaction mixture in the range of 1 mg per mL to 5 mg per mL of protein in the enzyme preparation (Appendices 3a and 3b).

Effect of Polyoxin D

Plots of reaction velocity (v) against substrate concentration (s) in Lineweaver-Burk coordinates (Figures 2a and 2b) in the presence of polyoxin D show that the pyrimidine antibiotic is a competitive inhibitor with respect to UDP-GlcNAc (i.e., K_m increased with respect to UDP-GlcNAc while V_{max} remained unchanged). The plots were linear with correlation coefficients (r), between 0.95 and 0.99. The apparent K_m as estimated by the double reciprocal plot (1/v against 1/[s]) was 2.0 mM and 1.8 mM for M. pusilla and M. candelabrum, respectively. The inhibition constant (K_i) was determined according to the Dixon method. A plot of polyoxin D concentrations against the reciprocal of reaction velocity at two substrate concentrations yielded an apparent K_i (constant of competitive inhibition with respect to UDP-GlcNAc) of 6.9 μM for M. pusilla and 5.8 μM for M. candelabrum (Figures 3a and 3b). These concentrations are about 1000 times smaller than the K_m for UDP-GlcNac. More than 90% inhibition was obtained at 5.0×10^{-5} M of polyoxin D for both species (Figure 4). The kinetic constants so determined are of the same order of magnitude as those obtained for other fungi species.

pH Optimum

KH_2PO_4/NaOH buffer at a final concentration of 25 mM was used to vary the pH of the reaction mixture. The optimum pH for the enzyme preparation of M. candelabrum was 6.2 and that of M. pusilla was 6.0 (Figure 5). Half maximum activity was obtained at a pH of 6.7 for M. pusilla and 6.9 for the M. candelabrum.

Figure 2a.

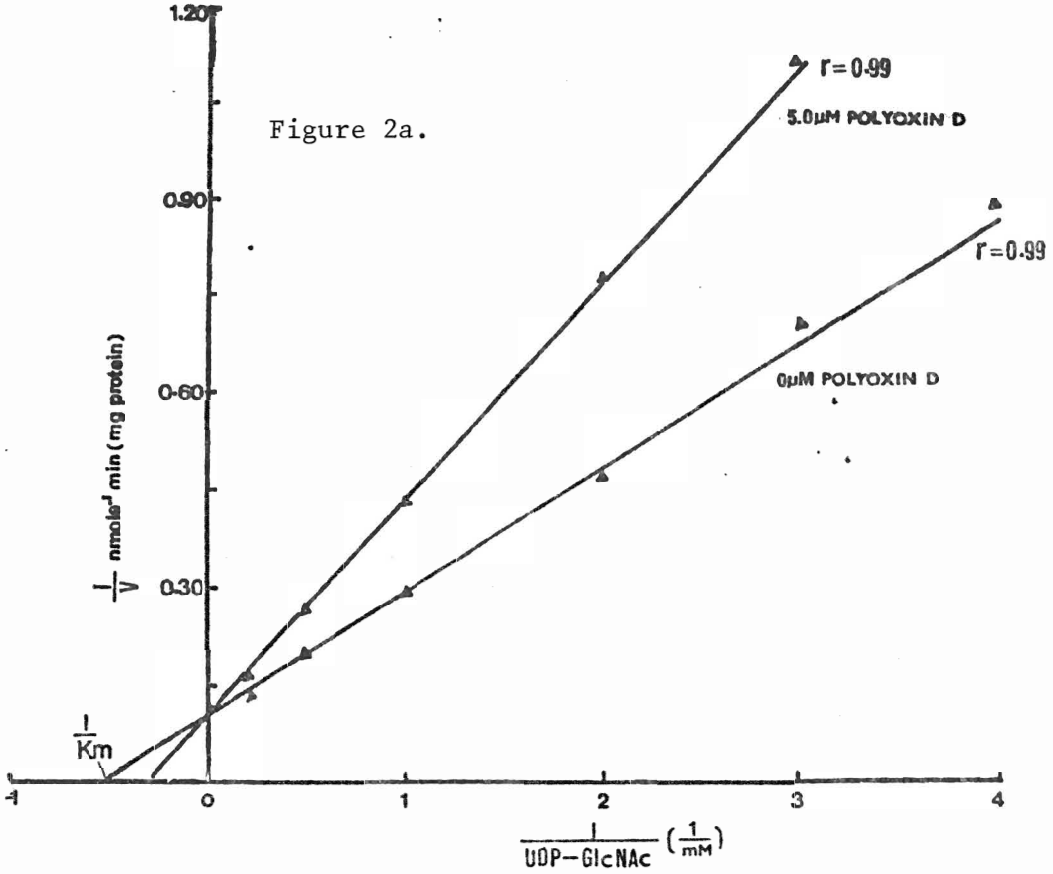


Figure 2b.

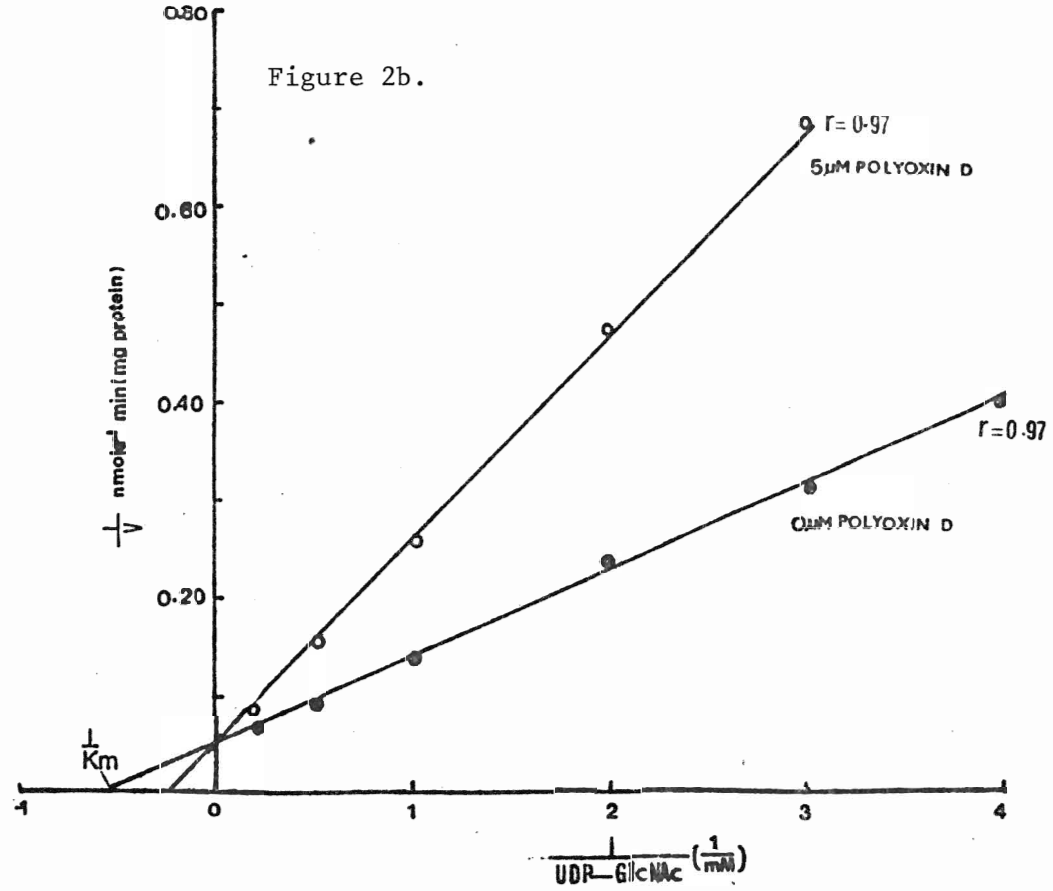


Figure 3a. Kinetics of inhibition of chitin synthase activity in mixed membrane fractions of M. candelabrum by Polyoxin D.

Data show a Dixon plot of enzyme activity with respect to polyoxin D at 2 mM and 5 mM UDP-GlcNAc. Chitin synthase activity was assayed as described under "Materials and Methods" in the presence of varying concentrations of polyoxin D. Mixed membrane preparations used were activated with acid protease (1000 μg mL^{-1} of enzyme) for 30 min. V is the incorporation rate of substrate into chitin (nmole per min per mg protein). Enzyme concentration: 5.1 mg per mL.

Figure 3b. Kinetics of inhibition of chitin synthase activity in mixed membrane fractions of M. pusilla by Polyoxin D.

Procedure and conditions were the same as described for M. candelabrum (Figure 3a). Enzyme concentration: 5.0 mg per mL.

Figure 3a.

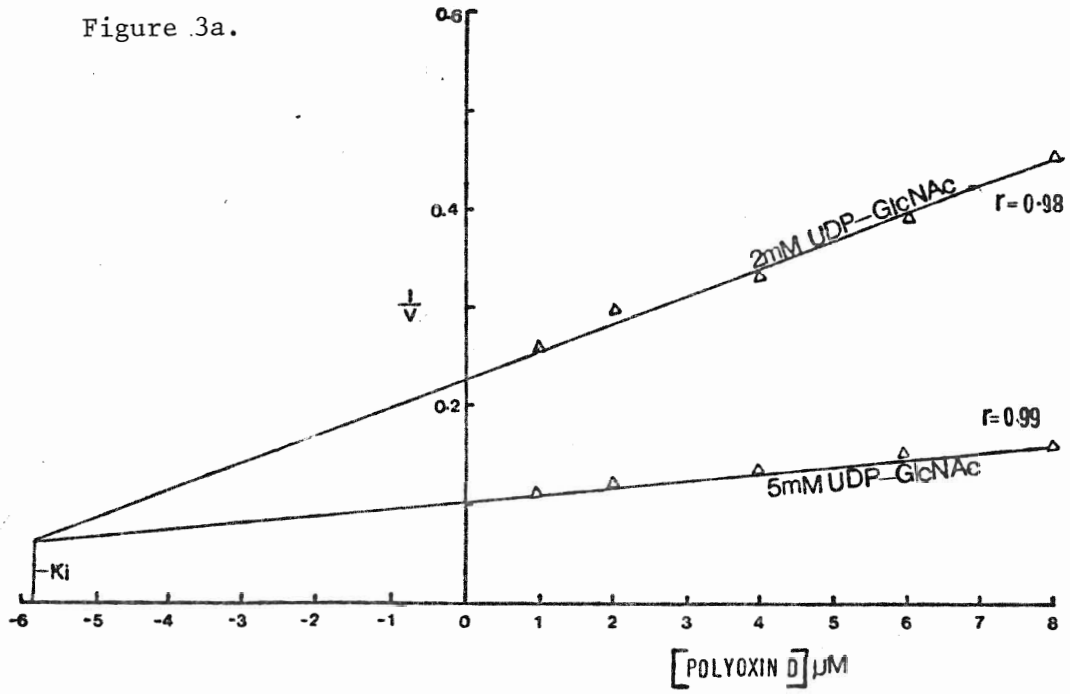


Figure 3b.

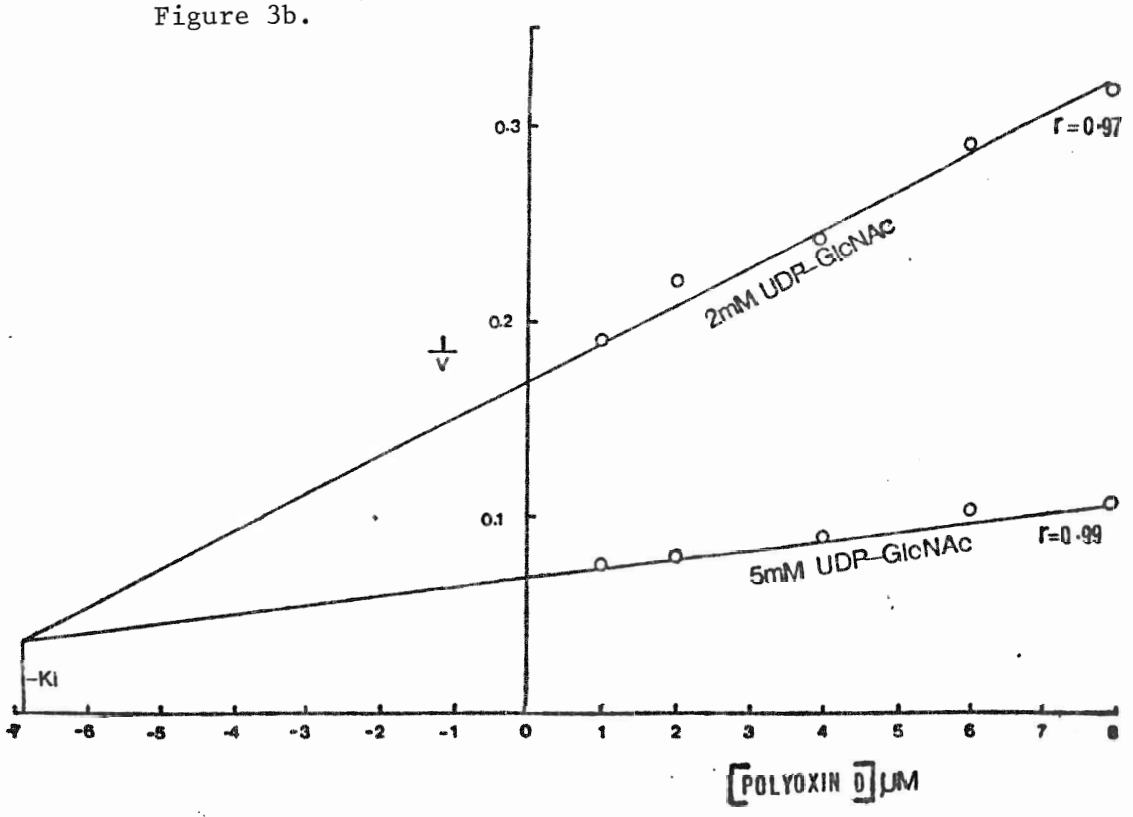


Figure 4. Inhibition of chitin synthase activity in mixed membrane fractions of M. candelabrum and M. pusilla by Polyoxin D.

Chitin synthase was assayed by the standard procedure described under "Materials and Methods". Varying amounts of polyoxin D were added to the assay mixture containing 0.5 mM UDP-GlcNAc. The reaction was started by adding acid protease (1000 μg mL^{-1}) activated mixed membrane fractions to the reaction mixture. Conditions were the same as described in the standard assay procedure. Enzyme concentration: M. candelabrum (Mc), 44 mg mL^{-1}; M. pusilla (Mp), 4.6 mg mL^{-1}

Figure 4.

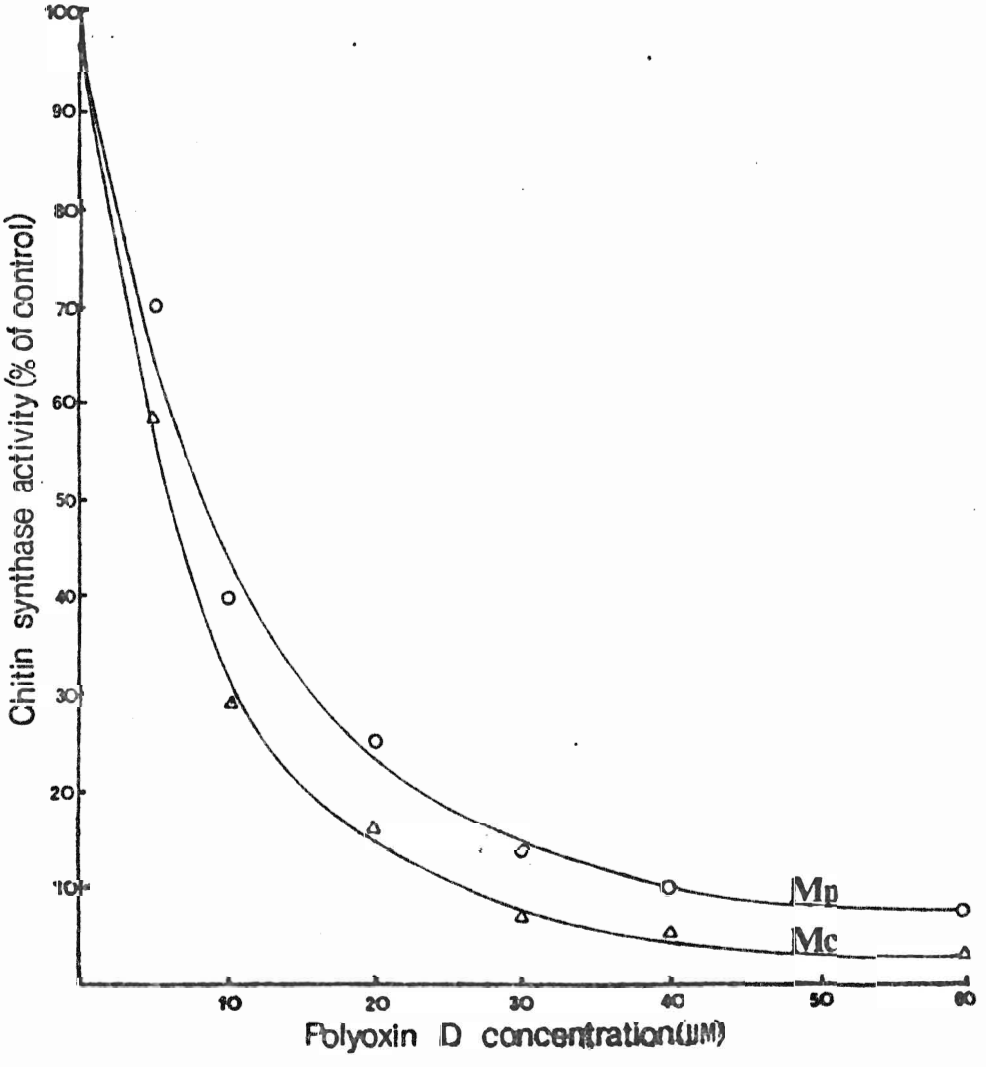
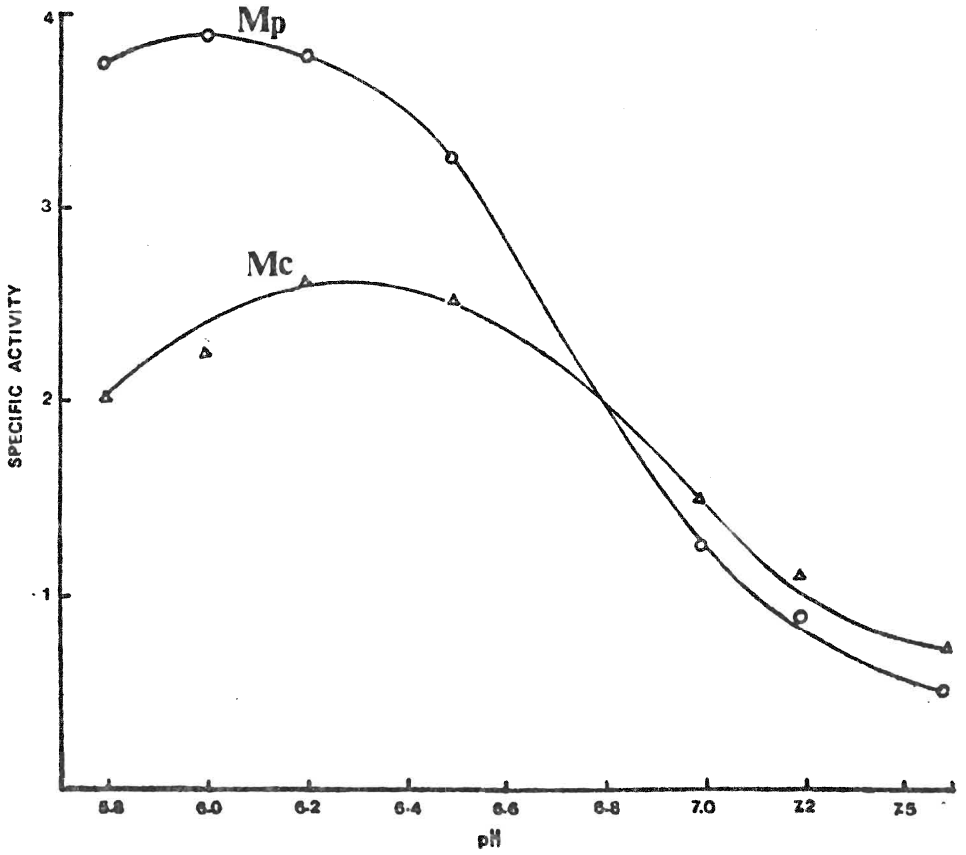

Chitin synthase activity (% of control)
100
90
80
70
60
50
40
30
20
10
Mp
Mc
10
20
30
40
50
60
Polyoxin D concentration(μM)

Figure 5. Effect of pH on chitin synthase activity.

Conditions for assay and concentration of reactants was the same as described in the standard assay procedure, except that the pH of the assay mixture was varied with KH_2PO_4/NaOH buffer at a final concentration of 25 mM. The reaction was started by adding acid protease (1000 μg mL^{-1}) activated membrane fractions to the standard assay mixture. Mixed membrane fractions were prepared as described under "Materials and Methods" and suspended in KH_2PO_4/NaOH (25 mM) buffer of pH 7.0. Enzyme concentration: 5.0 mg mL^{-1} (Mc); 5.0 mg mL^{-1} (Mp).

Figure 5.



Temperature

To investigate the effect of temperature on chitin synthase activity, the assay mixture was incubated at different temperatures in the range of 15° to 37°C. The highest enzyme activity was obtained at 28°C for M. pusilla and 30° for the enzyme of M. candelabrum (Figure 6).

Metal cation requirement

Stimulation of chitin synthase activity of both species by Mg^{+} was largely increased after acid protease treatment of the enzyme preparation (Figure 7). There was hardly any enzyme activity in the absence of Mg^{+2}, when non-activated enzyme preparations were used for chitin synthase assay. Only 4% of the maximum enzyme activity was obtained in the absence of Mg^{+2} with the non-acetivated mixed membrane fractions from the two species. Enzyme activity in the absence of Mg^{+2} was 20% of the maximum obtained with the activated enzyme of M. pusilla. In both species, chitin synthase activity increased with the increase in Mg^{+2} concentration in the reaction mixture, and reached a maximum when the cation concentration was between 25 mM and 35 mM. The effect of Mg^{+2} on activity in the non-activated enzyme fraction of M. candelabrum (unlike a similarly treated fraction from M. pusilla) was very gradual.

The stimulation of chitin synthase activity was found to be very dependent on the presence of divalent cations. The effect was relatively non-specific for a number of salts tested. Assays in which $MgCl_2$ was replaced by a range of other metal chlorides at 10 mM concentration showed

that Mn^{+2} and Co^{+2} can completely replace Mg^{+2} (Figure 8). Calcium was less effective in activating the enzyme activity. K^{+} and NH_4^{+} were essentially inert whereas iron (Fe^{+3}) and zinc inhibited chitin synthase activity. As can be seen in Figure 9, the range for maximum activation was rather broad and varied according to the cation.

The effect of N-acetylglucosamine on enzyme activity

Chitin synthase was activated by N-acetylglucosamine (GlcNAc). Maximum activation was obtained at a concentration of 50 mM GlcNAc for both species (Figure 10). As observed with Mg^{+2}, there was negligible enzyme activity in the absence of GlcNAc in the non-activated mixed membrane preparation from both species. As shown in Table 1, the non-activated enzyme of M. pusilla and the protease-activated enzyme of M. candelabrum showed absolute requirement for both Mg^{+2} and GlcNAc. Enzyme activity was very low in the absence of either activator. The stimulation effect of Mg^{+2} and GlcNAc seems to be additive for the activated enzyme of M. pusilla with each activator contributing about 50% of the newly activated enzyme activity. Heating the mixed membrane preparations resulted in a rather drastic decrease (75%) in the chitin synthase activity of M. candelabrum. Conversely, there was an increase (10%) in activity of the enzyme from M. pusilla upon similar treatment (heating at 45°C for 10 min). This increase did not offset the ratio of the total enzyme activity contributed by either Mg^{+2} or GlcNAc.

Figure 6. Effect of temperature on chitin synthase activity in mixed membrane fractions of M. pusilla and M. candelabrum.

Chitin synthase was assayed as described under "Materials and Methods" except that the assay temperature was varied as shown in the figure. Enzyme preparations from both species were pre-incubated for 30 min with acid protease (1000 μg mL^{-1}) before being added to the assay mixture at the start of the reaction. Enzyme concentration: 4.2 mg mL^{-1} (Mc); 4.5 mg mL^{-1} (Mp).

Figure 6.

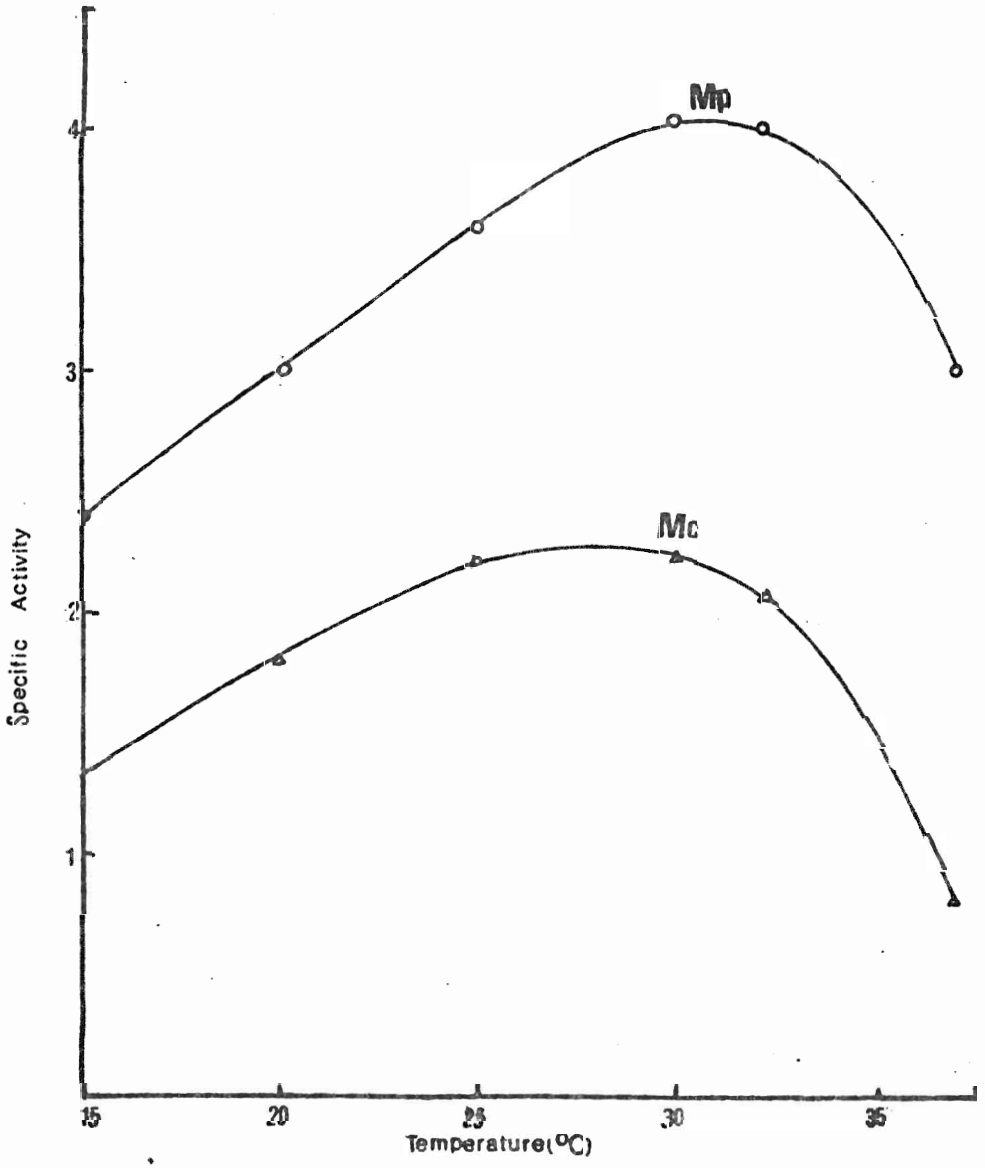


Figure 7. Effect of Magnesium on chitin synthase activity in non-activated and acid protease activated membrane fractions.

Chitin synthase was assayed under the standard incubation conditions described earlier, except that $MgCl_2$ concentration in the assay mixture was varied. Where indicated mixed membrane preparations were pre-incubated with acid protease (1000 $\mu g\ mL^{-1}$) for 30 min. Enzyme concentration: 5.2 $mg\ mL^{-1}$ (Mc); 5.0 $mg\ mL^{-1}$ (Mp).

Figure 7.

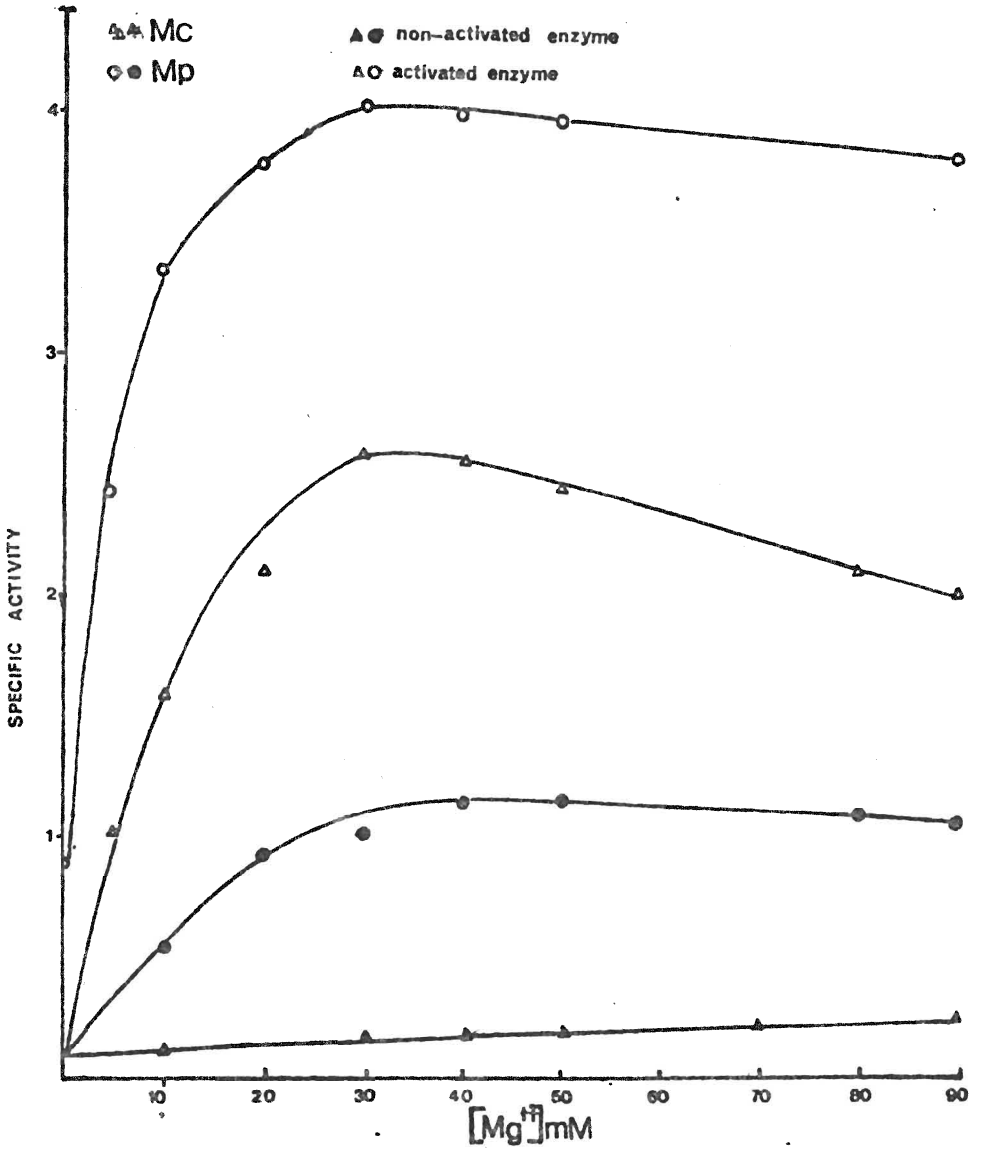

Mc
Mp
non-activated enzyme
activated enzyme
SPECIFIC ACTIVITY
1
2
3
4
10
20
30
40
50
60
70
80
90
[Mg++]mM

Figure 8. Effect of cations on chitin synthase activity.

Chitin synthase was assayed under conditions similar to those described under "Materials and Methods" except that where indicated $MgCl_2$ was replaced by chlorides of the various cations at 10 mM final concentration. Enzyme activity was determined in acid protease activated mixed membrane fractions. Control experiments to which no cation had been added were included. Enzyme concentration: 4.6 mg mL^{-1} (Mc); 4.9 mg mL^{-1} (Mp).

Figure 8.

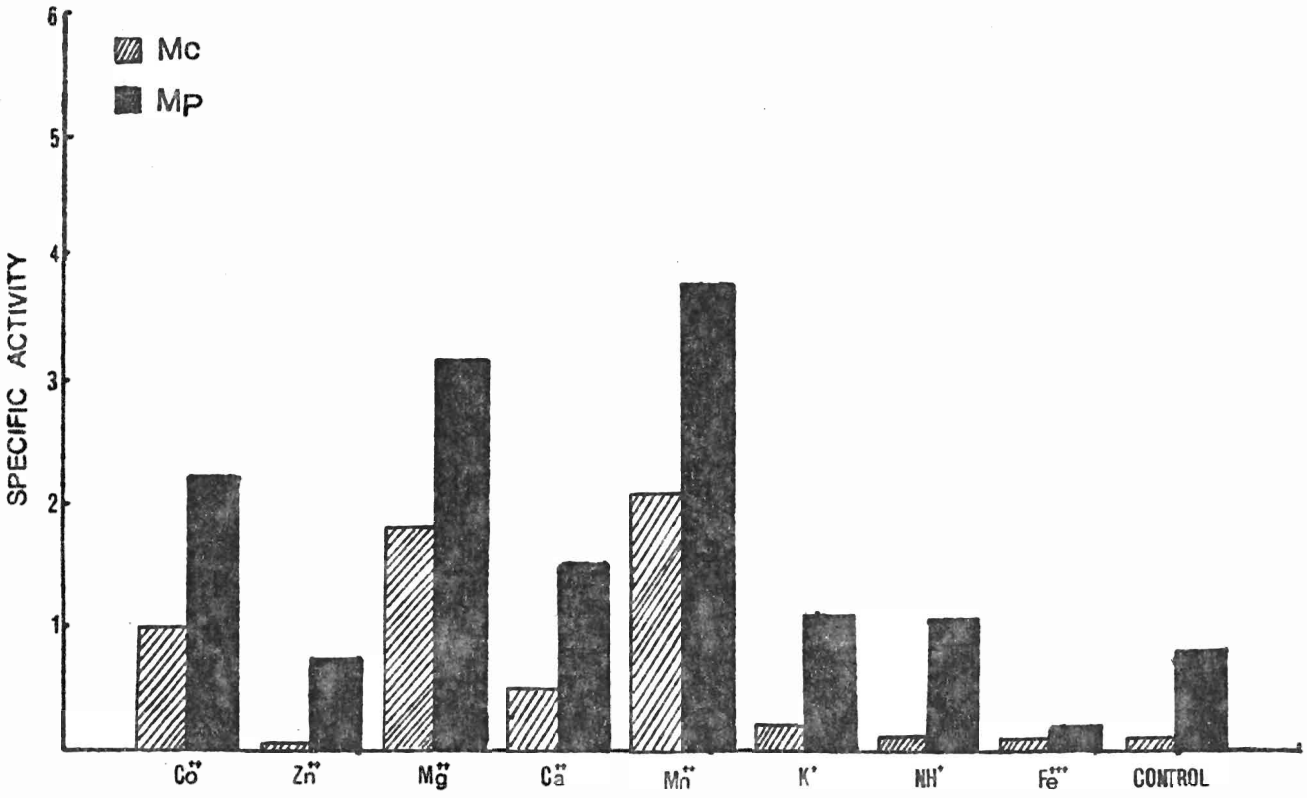


Figure 9. Effect of metal cations on chitin synthase activity.

Chitin synthase was assayed in mixed membrane fractions prepared as described under "Materials and Methods" by the standard assay procedure except that the concentration of divalent cations (Mg^{+2}, Mn^{+2} or Co^{+2}) added as chlorides was varied. Enzyme preparations used were pre-incubated with acid protease (1000 $\mu g\ mL^{-1}$) for 30 min. Enzyme concentration: 5.2 mg mL^{-1} (Mc); 5.3 mg mL^{-1} (Mp).

Figure 9.

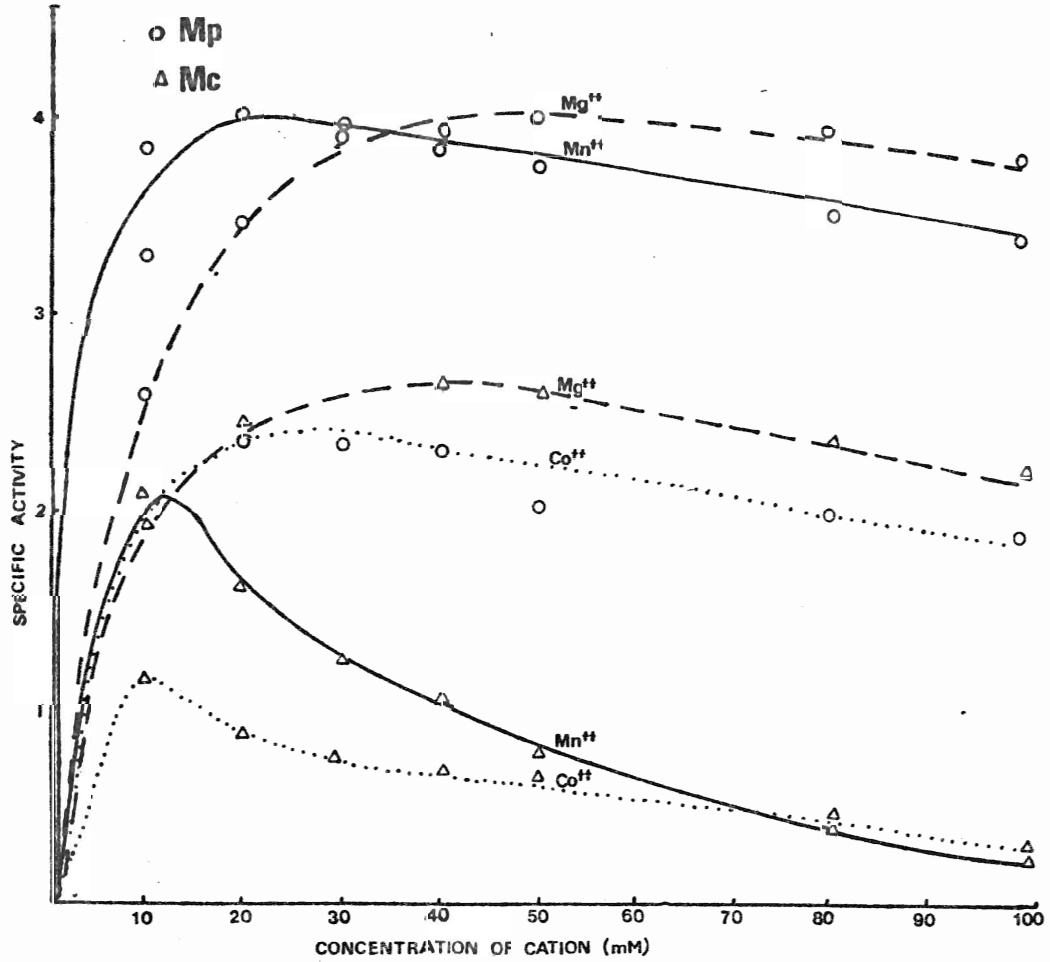

o Mp
Δ Mc
Mg++
Mn++
Mg++
Co++
Mn++
Co++
SPECIFIC ACTIVITY
1
2
3
4
10
20
30
40
50
60
70
80
90
100
CONCENTRATION OF CATION (mM)

Figure 10. Activation of chitin synthase by N-acetylglucosamine (GlcNAc) in *M. candelabrum* and *M. pusilla*.

Enzyme assay was carried out under the conditions described in the "Materials and Methods". Reaction mixtures contained various concentrations of GlcNAc with UDP-GlcNAc at a final concentration of 0.5 mM. Enzyme activity was assayed in both non-activated and acid protease (1000 μg mL^{-1}) activated mixed membrane fractions. Enzyme concentration: 4.2 mg mL^{-1} (Mc); 4.4 mg mL^{-1} (Mp).

Figure 10.

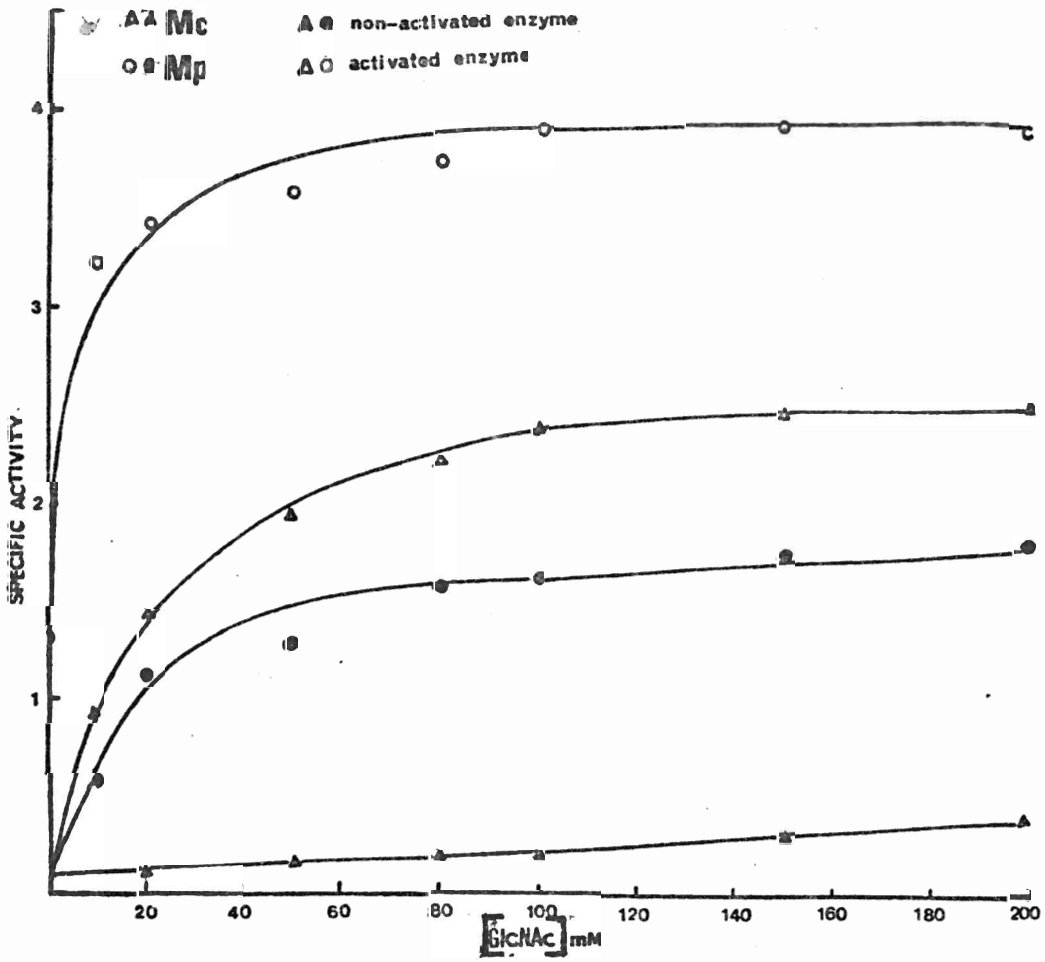

Mc
Mp
non-activated enzyme
activated enzyme
SPECIFIC ACTIVITY
1
2
3
4
20
40
60
80
100
120
140
160
180
200
[GlcNAc] mM

Table 1. Effect of Mg^{+2} and GlcNAc on chitin synthase activity.

The standard chitin synthase assay described in the "Materials and Methods" was used, except that where indicated the reaction was carried out in the absence of either $MgCl_2$ or GlcNAc or both. In a separate experiment, chitin synthase assay was carried out on mixed membrane preparations heated to 45°C for 10 minutes. The final concentrations of Mg^{+2} and GlcNAc in the assay mixture were as described earlier in the standard assay mixture.

Chitin Synthase Activity (nmole GlcNAc/min mg protein)

	Non-activated enzyme			
	M. candelabrum		*M. pusilla*	
Assay conditions	non-heated	heated	non-heated	heated
+ Mg^{+2} + GlcNAc	0.15	-- *	1.29	1.65
+ Mg^{+2} - GlcNAc	0.13	--	0.14	--
- Mg^{+2} + GlcNAc	0.10	--	0.11	--
- Mg^{+2} - GlcNAc	0.10	--	0.09	--
	Activated enzyme			
	M. candelabrum		*M. pusilla*	
	non-heated	heated	non-heated	heated
+ Mg^{+2} + GlcNAc	2.25	0.60	4.27	4.86
+ Mg^{+2} - GlcNAc	0.11	--	1.37	1.67
- Mg^{+2} + GlcNAc	0.06	--	1.20	1.84
- Mg^{+2} - GlcNAc	0.07	--	--	--

* "--" not determined.

Proteolytic activation

Chitin synthase is synthesized as a proenzyme (Cabib and Farkas, 1971), consequently, the expressed activity (i.e., the pre-activated activity found soon after cell disruption), and the total activity (i.e., the protease activated activity) in both species were assayed. Figure 11 shows the activity of chitin synthase in the mixed membrane fraction in the presence of varying concentrations of three proteases. In its native state (i.e., activity in the absence of protease treatment), the enzyme of M. candelabrum showed an especially low activity (<10% of the maximum), apparently being present in a zymogenic or inactive state requiring proteolytic activation (as is known for chitin synthase from other fungi). The membrane preparations were pre-incubated with proteases at concentrations indicated in Figure 11 before being added to the assay mixture at the start of the reaction. The responsiveness to proteases displayed specificity inasmuch as an acid protease (from Aspergillus saitoi) was an excellent activator, with 500-1000 μg mL^{-1} of enzyme preparation, producing more than 15-fold increase in the enzyme activity of M. candelabrum, and an increase of between two- and four-fold in that of M. pusilla (Appendix 5). Trypsin from bovine pancrease) and a neutral protease (from Bacillus amyloliqueficiens). elicited only a moderate degree of activation which, in addition, was limited to a relatively narrow concentration range (5 to 10 μg mL^{-1} of enzyme preparation). At higher concentrations, neutral protease and trypsin inhibited chitin synthase activity. The activation values in Appendix 5 were calculated from the highest response attained by the different proteases in

the same chitin synthase preparation. In all the experiments, the final pH was 6.2. This should be taken into consideration in assessing the relative effectiveness of the proteases.

Statistical analysis revealed that there was a significant difference in the total chitin synthase activity in the mixed membrane fractions of the two species, at acid protease treatment concentrations between 500 and 1000 $\mu g\ mL^{-1}$ (t test, $P < 0.001$). However, there was no significant difference in the response to activation between the two species (t test, $P > 0.2$) at the same concentration of acid protease. The quantity of inactive enzyme in both M. pusilla and M. candelabrum was essentially the same.

Chitin synthase activity during storage

When mixed membrane fraction from the two species, prepared as described under "Materials and Methods", were stored at 4°C, chitin synthase activity of M. pusilla was increased 4-fold over 48 hours to the level of activation achieved with the exogenous acid protease from Aspergillus saitoi (Figure 12). There was a negligible increase in the enzyme activity in the same fraction of M. candelabrum stored under similar conditions. The exogenously activated mixed membrane preparation from M. pusilla showed a relatively small increase in activity (20% increase) compared to the non-activated fraction over the same period of storage. The activated fraction of M. candelabrum showed a decline in activity to the level of the non-activated enzyme, i.e., about 90% decrease in maximum activity.

Figure 11.

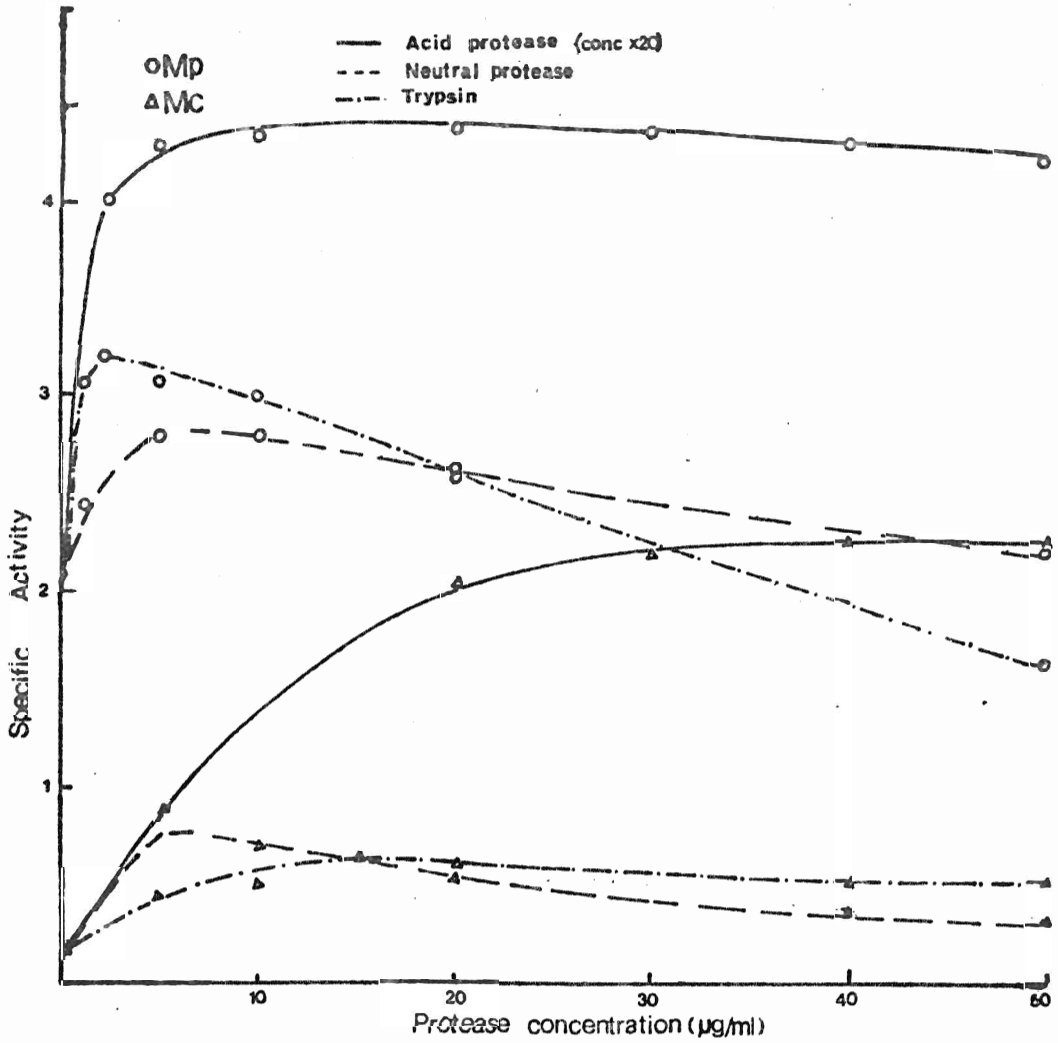
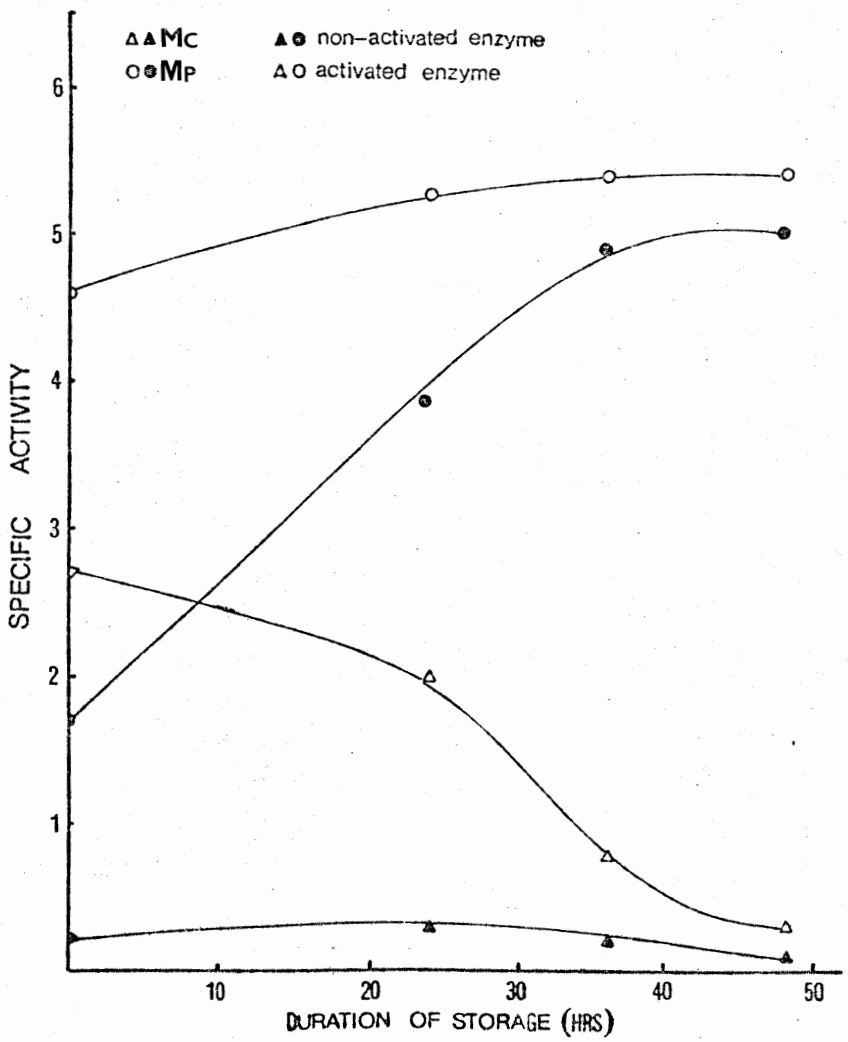

Acid protease (conc x20)
Neutral protease
Trypsin
oMp
ΔMc
Specific Activity
1
2
3
4
10
20
30
40
50
Protease concentration (μg/ml)

Figure 12. Chitin synthase activity in mixed membrane fractions of *M. candelabrum* and *M. pusilla* during storage.

Membrane fractions were prepared as described under "Materials and Methods" and stored at 4°C in KH_2PO_4/NaOH (25 mM) buffer, pH 7.0. Enzyme activity was assayed using the standard procedure over 48 hours. One mL samples of enzyme preparation were withdrawn at the time intervals indicated in the graph and chitin synthase activity measured at 30°C for 5 min. Enzyme activity was determined in both non-activated and acid protease (1000 μg mL^{-1}) activated fractions of the stored membrane preparation. Enzyme concentration: 4.8 mg mL^{-1} (Mc); 5.0 mg mL^{-1} (Mp).

Figure 12.


ΔAMc
OOMP
AA non-activated enzyme
AO activated enzyme
SPECIFIC ACTIVITY
1
2
3
4
5
6
10
20
30
40
50
DURATION OF STORAGE (HRS)

Cytoplasmic inhibitor of chitin synthase

The presence of a soluble cytoplasmic inhibitor of chitin synthase activity was observed in in vitro experiments in which lyophilized 100,000 g supernatant fractions resuspended in 25 mM KH_2PO_4/NaOH buffer, pH 7.0, were either pre-incubated with the mixed membrane preparations from the two species before assay or added to the assay mixture just before the assay. Wherever indicated in Table 2, the acid protease activated membrane fraction was further treated with the inhibitor fraction.

As can be seen from Table 2, incubation with the cytoplasmic fraction of M. candelabrum produced a strong inhibition (75%) of the chitin synthase activity in its activated mixed membrane fraction. The inhibition of chitin synthase activity was much lower (28%) in a similarly treated membrane fraction of M. pusilla. A higher degree of inhibition (60%) was obtained when the non-activated membrane preparation of M. pusilla was treated with its cytoplasmic fraction. The corresponding inhibition of chitin synthase activity in the mixed membrane preparation of M. candelabrum was only 28%. The cytoplasmic inhibitor of M. pusilla seems to be more effective against the pre-activated enzyme, whereas that of M. candelabrum produced the greatest inhibition against the acid protease activated chitin synthase activity. The exogenous protease used was of Aspergillus saitoi origin and this should be considered in determining the mechanism of action of the cyotplasmic inhibitor.

Table 2. Effect of supernatant inhibitor on chitin synthase activity in mixed membrane preparations of M. candelabrum and M. pusilla.

Chitin synthase activity was assayed under the same conditions as described in the "Materials and Methods". The 100,000 g supernatant fraction was lyophilized and resuspended in the homogenizing buffer (KH_2PO_4/NaOH, pH 7.). Where indicated the mixed membrane fraction from each species was either pre-incubated with its supernatant fraction (100 µL mL^{-1} of enzyme suspension) for 10 min or with acid protease (1000 µg mL^{-1}) for 30 min. before the addition of substrate (activation stage). In other assays, the supernatant fraction was added during the incubation of enzyme with substrate (assay stage) after activation of the enzyme with acid protease. Protein concentration in MMF, 3.7 mg mL^{-1} (Mp); 3.9 mg mL^{-1} (Mc); SF, 24.5 mg mL^{-1} (Mp); 22.0 mg mL^{-1} (Mc).

addition during activation stage		addition during assay stage	[^{14}C]-GlcNAc incorporated nmole/min mg protein			
cytosolic inhibitor	acid protease	cytosolic inhibitor	Mp	inhibition (%)	Mc	inhibition (%)
- *	-	-	1.59		0.17	
+ **	-	-	0.65	60	0.13	28
-	-	+	0.72	56	0.11	35
-	+	-	4.05		1.95	
-	+	+	2.92	28	0.49	75
+	+	-	3.22	20	0.51	74

* "-" no addition

** "+" addition

Endogenous protease activity

The presence of endogenous proteases in the mycelial extracts of both species was determined as described in the "Materials and Methods". Substrate specificity was tested using casein, hemoglobin and a synthetic substrate (Nα-benzoyl-DL-arginine-p-nitroanilide, BAPA). The results in Table 3 show that there was more protease activity associated with the mixed membrane preparation of M. pusilla than that of M. candelabrum when both casein and hemoglobin were used as substrates. Conversely, the protease activity in the supernatant fraction of M. candelabrum was much higher than that of M. pusilla. Neither fraction, however, showed any detectable protease activity when the synthetic substrate was used. The protease of both species showed some characteristics of serine proteases, namely inhibition by phenylmethylsulfonylfluoride (PMSF), (results not shown), though the inhibition was not strong.

Table 3. Proteolytic activity in mixed membrane and supernatant fractions of *M. candelabrum* and *M. pusilla*.

Samples of mixed membrane preparations in phosphate buffer, pH 7.0, or high speed supernatant fraction were incubated with acid-denatured hemoglobin, casein or synthetic substrate (DL-BAPA). Proteolytic activity was measured as the tyrosine and tryptophan content of the soluble products of hydrolysis of the substrates as described under "Materials and Methods" and expressed either as TU^{cas} or as µmole of soluble tyrosine (mg protein)$^{-1}$ h^{-1}. [One TU^{cas} unit is the amount of trypsin which under defined conditions (20 min incubation at 35°C, final volume of the incubation mixture: 2.0 mL; after addition of trichloroacetic acid: 5.0 mL) liberates sufficient trichloroacetic acid soluble hydrolysis products so that the extinction at 280 nm increases by 1.00 in 1 min. Specific activity was expressed as TU^{cas} per mg protein.] Mc--*M. candelabrum*; Mp--*M. pusilla*

	Endogenous protease activity with		
	casein	hemoglobin	DL-BAPA**
Subcellular fraction	TU^{cas} / mg protein	µmole tyrosine / mg protein h	
mixed membrane fraction (Mp)	54.6	27.6	--*
mixed membrane fraction (Mc)	45.2	19.2	--
supernatant fraction (Mp)	20.3	11.7	--
supernatant fraction (Mc)	33.0	22.5	--

* "--" not measurable
** BAPA: Nα-benzoyl-DL-arginine-p-nitroanilide

DISCUSSION AND CONCLUSION

Cell-free extracts from mycelial homogenates of Mortierella candelabrum and Mortierella pusilla were found to catalyse the transfer of N-acetyl-D-glucosamine from uridine diphosphate N-acetyl-D-glucosamine (UDP-GlcNAc) to chitin. The reaction product, chitin, a β-(1-4)-linked polymer of N-acetylglucosamine was identified mainly on the basis of the compounds released after acid or enzymatic hydrolysis. Identification of the chromatographically immobile substance as chitin was confirmed by its hydrolysis with concentrated hydrochloric acid to glucosamine and the action of chitinase upon it. The fact that chitinase caused a substantial breakdown of the reaction product and only the dimer diacetylchitobiose was found in appreciable amounts from the chitinase digest, shows that the transferred acetylglucosamine was linked to another residue of the same monosaccharide. The insolubility of most of the polysaccharide after alkali or acetic acid digestion is also in agreement with the properties of chitin.

With regard to the cellular location of chitin synthase, the results clearly show that about 80% of the total activity was sedimented with the mixed membranes. The remaining 20% was in the cell wall and the 100,000 g supernatant fractions. The chitin synthase found in the cytosolic fraction probably represents nascent enzyme prior to its migration to the cell wall, whereas the cell wall bound chitin synthase

represents enzyme at its site of action or contamination from the membranous fraction. Studies on chitin synthase in protoplasts of other fungi which is still in progress (Bartnicki-Garcia, 1973; de Vries and Wessels, 1975) will help eludicate the nature of the relationship between the enzyme and the hyphal wall.

The chitin synthase from both M. candellabrum and M. pusilla resembles those of other fungi in its general requirements. Thus, a divalent cation was necessary for the enzyme reaction and N-acetylglucosamine increased the activity of the enzyme. The antibiotic, polyoxin D, was a potent competitive inhibitor with respect to the substrate (UDP-GlcNAc). Among the various metal cations tested, Mg^{+2}, Co^{+2} and Mn^{+2} largely stimulated the enzyme activity. Other cations such as Ca^{+2}, were less effective, while Zn^{+2} was inhibitory. This observation might suggest that the activation effect of Mg^{+2}, Co^{+2} and Mn^{+2} was interchangeable. There must, however, be reservations about the exact role of divalent cations in the in vitro activation of chitin synthase. The intracellular concentration of divalent cations in the two species is not known; consequently it is not known whether changes in the activity of the enzyme can be induced by alternation of intracellular cation concentration. It seems reasonable to assume that divalent cations, preferable Mg^{+2}, Co^{+2} and Mn^{+2}, are essential cofactors for the activity of chitin synthase, but the role for these cations as physiological regulators of the enzyme has yet to be firmly established. The increase in enzyme activity by free N-acetylglucosamine (GlcNAc) has also been recorded for the chitin synthase from other fungi (Glaser and

Brown, 1957; Potter and Jaworski, 1966; Gooday and de Rousset-Hall, 1975). The role of free GlcNAc in the reaction is not apparent. Porter and Jaworski, 1966, have shown that the free monomer is not incorporated into insoluble chitin. The substrate of the reaction, UDP-GlcNAc has been shown by Kornfeld et al. (1964) to be a feedback inhibitor in the pathway of GlcNAc synthesis. The activation by GlcNAc would, therefore, prevent premature feedback inhibition due to the build up of UDP-GlcNAc before the insoluble end product was accumulated to the required level. An allosteric effector role of GlcNAc has also been suggested (McMurrough and Bartnicki-Garcia, 1971; de Rousset-Hall and Gooday, 1975; Gooday, 1978). However, as relatively high concentrations of GlcNAc are needed to produce the same activation as produced by much lower concentrations of UDP-GlcNAc, Ruiz-Herrera et al. (1977) have suggested that UDP-GlcNAc is the natural effector and that GlcNAc simply mimics its effect. Although none of the possible mechanisms by which GlcNAc activates chitin synthase was investigated in the present study, the retainment of sigmoidal saturation kinetics in the presence of GlcNAc which has been the basis for its claim as an allosteric effector might suggest a far more complex function of the activator which may or may not include a positive effective role (Monod et al., 1963). The possibility has been considered (Carmago et al., 1971; McMurrough et al., 1971) that an additional stimulatory effect of GlcNAc on the in vitro biosynthesis of chitin might reside in its function as a primer for the initiation of new chains of chitin. However, this has found little experimental support (Gooday and de Rousset-Hall, 1975; Ruiz-Herrera

et al., 1977). Activation of chitin synthase by GlcNAc might, however, be of physiological significance. Gooday (1978) proposed that chitinase activity which softens the cell wall during cell wall synthesis, produces localized regions with elevated GlcNAc concentrations which activate chitin synthase. The considerable degree of activation of chitin synthase by Mg^{+2} and GlcNAc observed with membrane preparations from the two species further supports their cofactor and activator roles, respectively, in agreement with reports concerning other chitin synthases (cf. Gooday, 1978 and references therein). Some differences were noted between the two species in their response to Mg^{+2} and GlcNAc. Whereas the pre-activated enzyme of M. pusilla and the activated enzyme of M. candelabrum demonstrated absolute requirement for both Mg^{+2} and GlcNAc, the effect of either factor was additive for the activated enzyme of M. pusilla with each factor contributing about 50% of the activated activity of the enzyme. Low temperature heating resulted in an increase in enzyme activity but did not offset this ratio. This observation leads one to suggest that there might be two separate enzymes in M. pusilla which catalyse the synthesis of chitin. Further studies of the effect of these factors on the purified enzyme of M. pusilla should provide more insight into this suggestion.

In both fungi, chitin synthase in the cell-free extract exists in two states: "zymogenic" or inactive, and active. The relative amounts of these forms were estimated in assays with and without an exogenous protease. Between the two fungi, there was a significant difference in the total amount of chitin synthase (t test, $P < 0.001$). The total enzyme activity

in M. candelabrum was about 50% of that of M. pusilla. There was, however, no significant difference in the amount of inactive enzyme of both species (t test, $P > 0.2$). The zymogens of the two fungi responded similarly to neutral and acid proteases. The highest activation was achieved with the acid protease. The differences observed in the initial ratio of zymogen to active enzyme may not necessarily reflect actual in vivo differences in activation of chitin synthase in the two fungi but might have resulted mostly from differences in the nature and quantity of endogenous proteases released during cell breakage. These results further suggest that either the enzyme is produced in the cell as a true zymogen which is activated by breakage at a specific point in the molecule, or that the enzyme is masked by the presence of an inhibitory factor which is released by partial digestion. This is an attractive possibility in view of the observation in yeast (Cabib and Farkas, 1971; Cabib and Keller, 1971; Cabib and Ulane, 1973) that chitin synthase exists in an inactive form which can be converted by a protease to the active enzyme. The presence of a macromolecular material inhibiting the action of the protease has also been demonstrated by the same workers. The large increase in chitin synthase activity in the membrane fractions from M. pusilla during low temperature storage to levels of activation achieved with exogenous proteases suggests that the crude enzyme preparation of this species might contain some intrinsic factor capable of activating the enzyme. There was hardly any increase in activity of similarly treated fractions of M. candelabrum. Such an increase in chitin synthase activity during storage of M. rouxii

extracts at -20°C has been taken as evidence for proteolytic activation (McMurrough and Bartnicki-Garcia, 1973). Increases in protease activity on storage have also been observed in extracts from Phycomyces blakesleeanus (Fischer and Thomson, 1979), Saccharomyces cerevisiae (Neussdoerffer et al., 1980) and Aspergillus niger (Stevens et al., 1981). The increase in activity of proteases in freshly prepared crude extracts has been attributed to the presence of specific protease inhibitors which in the intact organism occur in a separate compartment but which in disrupted cell extracts are able to combine with the proteases. These inhibitors become digested when crude extracts are allowed to autolyse (Wolf and Holzer, 1980). As with the yeast protease system, the inhibitor of M. pusilla was probably digested by proteases during storage. In S. cerevisiae it has been shown that the proteases are present in the vacuoles whereas the inhibitors are present in the cytosolic fraction (Cabib 1975; Ulane and Cabib, 1974; Bunning and Holzer, 1979; Fischer and Holzer, 1980). Chitin synthase instability as observed in the stored activated mixed membrane preparation of M. candelabrum can be attributed to proteolysis but may not necessarily have any physiological significance. However, in vivo enzyme inactivation in response to physiological or developmental changes is frequently observed in microorganisms (Switzer, 1977). The evidence for a specific role of proteases in the activation of zymogen remains circumstantial. The role of inhibitors may be to regulate protease activity or to protect proteins from degradation should the vacuoles leak.

The present study revealed that the 100,000 g supernatant of both species inhibited chitin synthase activity. The active component in this crude extract has yet to be identified. This would require further fractionation of the cytosolic fraction. The inhibitor of M. pusilla was more effective against the pre-activated chitin synthase producing 60% inhibition. A much higher inhibition (about 75%) was obtained with the cytosolic fraction of M. candelabrum on its protease activated chitin synthase. Only 28% inhibition was observed in a similarly treated enzyme of M. pusilla. It appears from these results that the inhibitor of M. pusilla is more effective against the pre-activated (expressed) chitin synthase whereas that of M. candelabrum inhibited the activated enzyme. Lopez-Romero et al. (1978) isolated from extracts of M. rouxii a protein capable of inhibiting the in vitro synthesis of chitin. In contrast to the inhibitor of S. cerevisiae (Cabib and Farkas, 1971), the isolated substance of M. rouxii did not interfere with the proteolytic activation of the zymogen by the activating factor, but it directly inhibited the pre-activated chitin synthase. This pattern of inhibition is similar to that obtained in the M. pusilla system. The action of the inhibitor in M. candelabrum was different from that observed in S. cerevisiae in that the inhibitor of M. candelabrum seems to be able to reverse the stimulatory effect produced by the activator. It appears that the inhibitor of M. candelabrum neither inhibited the activating factor nor the pre-activated chitin synthase, but directly inhibited the activated enzyme. It should be noted, however, that the activator used in this study is an exogenous acid

protease from Aspergillus saitoi. This must be considered in assessing the role of the inhibitor on the activator or vice versa. The proteases of the two species might be acid in nature. This suggestion has been based not only on the observation that an exogenous acid protease was the most effective in activating the zymogen of the two species, but it has also been shown that fungal acid proteases are able to hydrolyse a range of native proteins, whereas the majority of them have little or no activity on synthetic substrates (Tanaka et al., 1977). Many acid proteases have been shown to be able to cleave phe(105)-met(106) bond of K-casein (Ichishima et al., 1980). The proteases of both M. pusilla and M. candelabrum had no measurable activity with the synthetic substrate (Nα-benzoyl-DL-arginine-p-nitroanilide) but both could hydrolyse casein. The endogenous protease activity in both species was inhibited to some extent by PMSF. It is, however, difficult to simply classify the enzymes as serine type proteases, because PMSF is also known to inhibit certain thiol proteases. When this is the case, inhibition can be reversed by 2-mercaptoethanol or dithiothreitol.

The pre-activated (expressed) chitin synthase activity of M. pusilla constitutes a large fraction (>50%) of the total enzyme activity. The suggestion from this study that this fraction of the enzyme is what is held in check by the cytoplasmic inhibitor seems to be reasonable. In M. candelabrum on the other hand the expressed activity constitutes a very small percentage (<10%) of the total chitin synthase activity. It is, therefore, proper that a control of the quantity of enzyme if any, should

be imposed on the activated enzyme. These are the controls which seem to be operative in the enzyme systems of the two species from the present *in vitro* investigation. Whichever way it acts, it seems that the inhibitor provides a safety device which regulates or localizes chitin synthase activity. It remains to be explained how the activation of chitin synthase occurs at a specific time or site. It seems this would require some subcellular organization which is not very well understood at this time. The higher protease activity observed in the mixed membrane fractions of *M. pusilla* further lends support to the possibility that endogenous proteases might have resulted in the increase in chitin synthase activity during storage. The high levels of protease activity in the cytosolic fraction of *M. candelabrum* coupled with the correspondingly higher levels of inhibitor in this fraction also support the suggestion made in this study that the soluble inhibitor of this species does not act on the protease activating factor but inhibits the activated chitin synthase directly. The protease activity detected in the mixed membrane fraction of *M. candelabrum* cannot be reconciled with the observation that there was no increase in chitin synthase activity in this fraction on storage. The basis of this observation is undoubtedly very complex, and three phenomena are thought might have contributed to the observed result:

(i) the activated enzyme was immediately acted upon by the inhibitor,

(ii) the protease could not release the inhibitor from the enzyme-inhibitor complex, and

(iii) the endogenous proteases demonstrated *in vitro* might not necessarily be the agents responsible for *in vivo* activation of the zymogen.

An investigation of these possibilities would have to await further characterization of the protease and inhibitor.

Irrespective of the molecular mechanism, the stimulation of chitin synthase by proteases and other factors is clearly an important potential regulatory system for the fine control of chitin synthesis in vivo. As the precise function of most proteases is unknown, a further study of the proteolytic enzymes of these two species will contribute to our understanding of their role in many cellular functions involving proteolysis, including activation of proenzyme and inactivation of specific enzymes. The difficulties associated with the preparation of fungal cell extracts by techniques which allow the isolation of intact organelles might limit the study of intracellular proteases. Further studies should be aimed at purifying the intracellular proteases from the mycelial extracts and thoroughly investigating their precise function from a biochemical standpoint. By separating and characterizing the proteases and studying their relationship to the inhibitor, the nature and function of the inhibitor will be understood properly. This knowledge about the proteases may also aid the development of methods which prevent artifacts caused by proteolysis during preparation of cellular materials. Conclusions that can be made about the physiological role of the proteases of the two species are limited, since physiological substrates were not used. Substrate specificity might indicate the range of proteolytic events in which a protease might be able to participate, but it cannot always be related directly to activity on the physiological substrates. Although this study does suggest a vital

function of proteases and inhibitors, most questions about their *in vivo* role remain speculative. A more direct approach is dependent on the ability to manipulate protease activity *in vivo*. Many inhibitors are now available and have been used in *in vivo* studies. However, not all are necessarily specific for individual proteolytic enzymes and there is a danger that processes other than those involving protease activity might be affected (Shechter *et al.*, 1973). In this respect, the streptomyces inhibitors such as pepstatin, antipain, leupeptin and chymostatin are of particular value, since side effects have not been reported. However, unless it is possible to demonstrate unequivocally that only one particular protease is inhibited by each agent, conclusions must be limited. A more precise means of manipulating protease activity *in vivo* is through the isolation of appropriate mutants (Achstetter *et al.*, 1981). It has been emphasized by Wolf (1982) that some proposals made on the basis of *in vitro* protease action have been shown to be incorrect as a result of examining mutant strains. Polyoxin D, a potent inhibitor of chitin synthesis, should be an ideal chemical for the selection of cell wall synthesis mutants of these species.

It is proposed that differences in the amount of total chitin synthase and the regulation of its activity is responsible for the differences observed in the pattern of cell wall construction in the two species. The high levels of chitin synthase in *M. pusilla* may account for its much thicker wall structure. The properties of chitin synthase activity in the two species suggests that *in vivo* a delicate balance exists between the

activation and inactivation of the enzyme which is responsible for the pattern of wall growth of each fungus. It is assumed that the factors mentioned in this study and perhaps others unknown, participate in a controlled proteolytic activation or inactivation of chitin synthase, depending on the physiological condition of the cell.

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Appendix 1. Properties of chitin synthase preparations from fungi.

Organism	Cell wall category*	"K_m" for UDP-GlcNAc mmole L^{-1}	Optimum pH	Optimum temperature	"K_1" for Polyoxin D µmol L^{-1}	Optimum $[Mg^{+2}]$ mmole L^{-1}	Reference
ZYGOMYCETES							
Mucor rouxii	chitin-chitosan	0.5-1.5	6.5	24-27	0.6	30	McMurrough and Bartnicki-Garcia (1971)
Mortierella candelabrum	chitin-chitosan	1.8	6.2	28	5.8	30	unpublished**
Mortierella pusilla	chitin-chitosan	2.0	6.0	30	6.9	30	unpublished**
Mortierella vinacea	chitin-chitosan	1.8	6.0	32	--	20	Peberdy and Moore (1975)
ASCOMYCETES							
Saccharomyces cerevisiae	mannan-glucan	0.6-0.9	6.2	37	0.5	10	Keller and Cabib (1971)
Neurospora crassa	chitin-glucan	1-2	7.5	27	1.4	--	Glaser and Brown (1957)
BASIDIOMYCETES							
Coprinus cinereus	chitin-glucan	0.9	8.0	30	3.0	30	Gooday and de Rousset-Hall (1975)

* from Bartnicki-Garcia (1970)
** present study

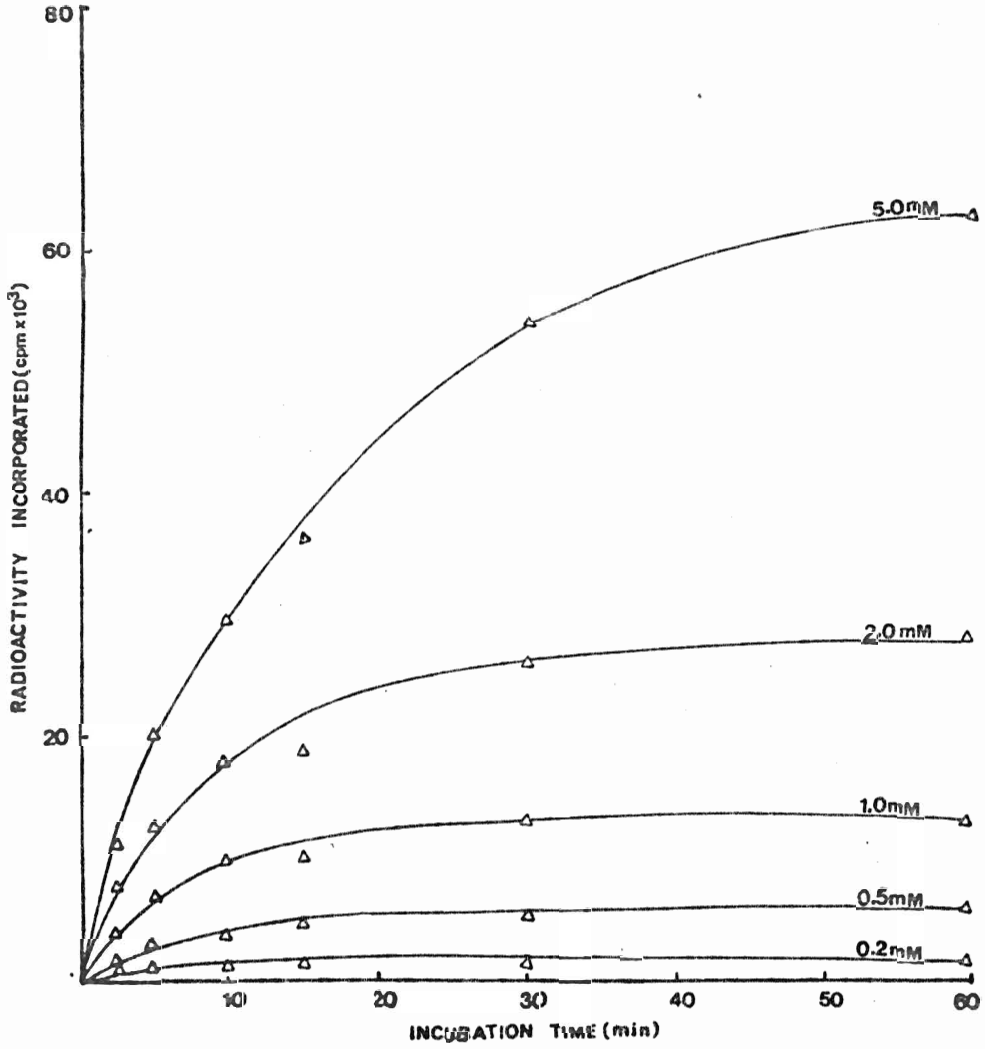
Appendix 2a. Dependence of chitin synthesis on time and substrate concentration in *M. candelabrum*.

Determination of chitin synthase activity was carried out by the standard procedure except that the concentration of UDP-GlcNAc and time of incubation were varied as indicated in the graph. Acid protease (1000 μg mL^{-1}) activated membrane preparations from both species were used. The reaction was stopped at the indicated times and chitin synthase activity was assayed as described under "Materials and Methods". Radioactivity incorporated (cpm) was plotted against incubation time (min). Enzyme concentration: 5.4 mg mL^{-1}.

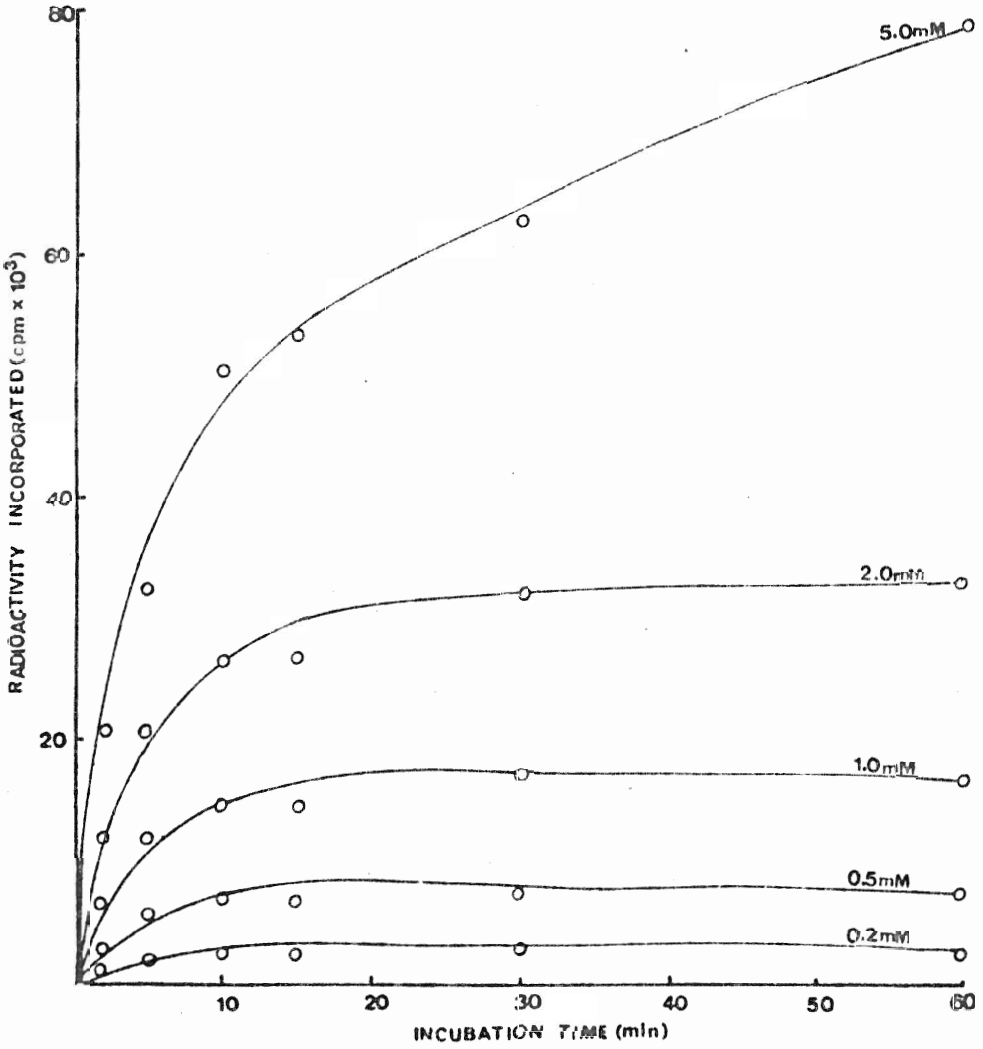
Appendix 2b. Dependence of chitin synthesis on time and substrate concentration in *M. pusilla*.

The experimental procedure was the same as described for *M. candelabrum*. Enzyme concentration, 5.1 mg mL^{-1}.

Appendix 2a.


80
60
40
20
RADIOACTIVITY INCORPORATED (cpm x 10³)
5.0mM
2.0mM
1.0mM
0.5mM
0.2mM
10
20
30
40
50
60
INCUBATION TIME (min)

Appendix 2b.


80
60
40
20
RADIOACTIVITY INCORPORATED (cpm x 10³)
5.0mM
2.0mM
1.0mM
0.5mM
0.2mM
10
20
30
40
50
60
INCUBATION TIME (min)

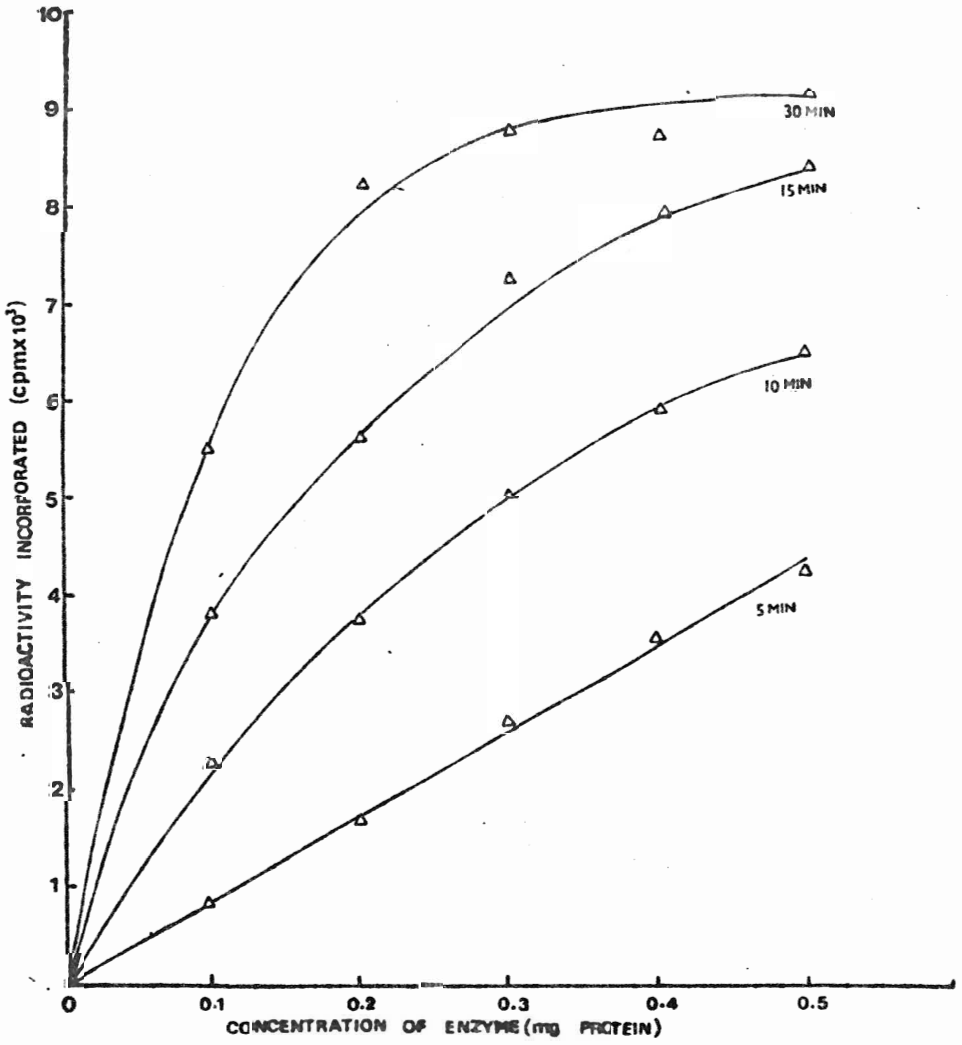
Appendix 3a. Effect of enzyme concentration on chitin synthesis in *M. candelabrum*.

Assay conditions were as described for the standard method except that chitin synthase activity was determined using various concentrations of enzyme (mixed membrane fraction) at different time intervals. The enzyme fraction was activated with acid protease (1000 μg mL^{-1}) for 30 min. Radioactivity incorporated (cpm) is plotted against concentration of enzyme (mg of protein).

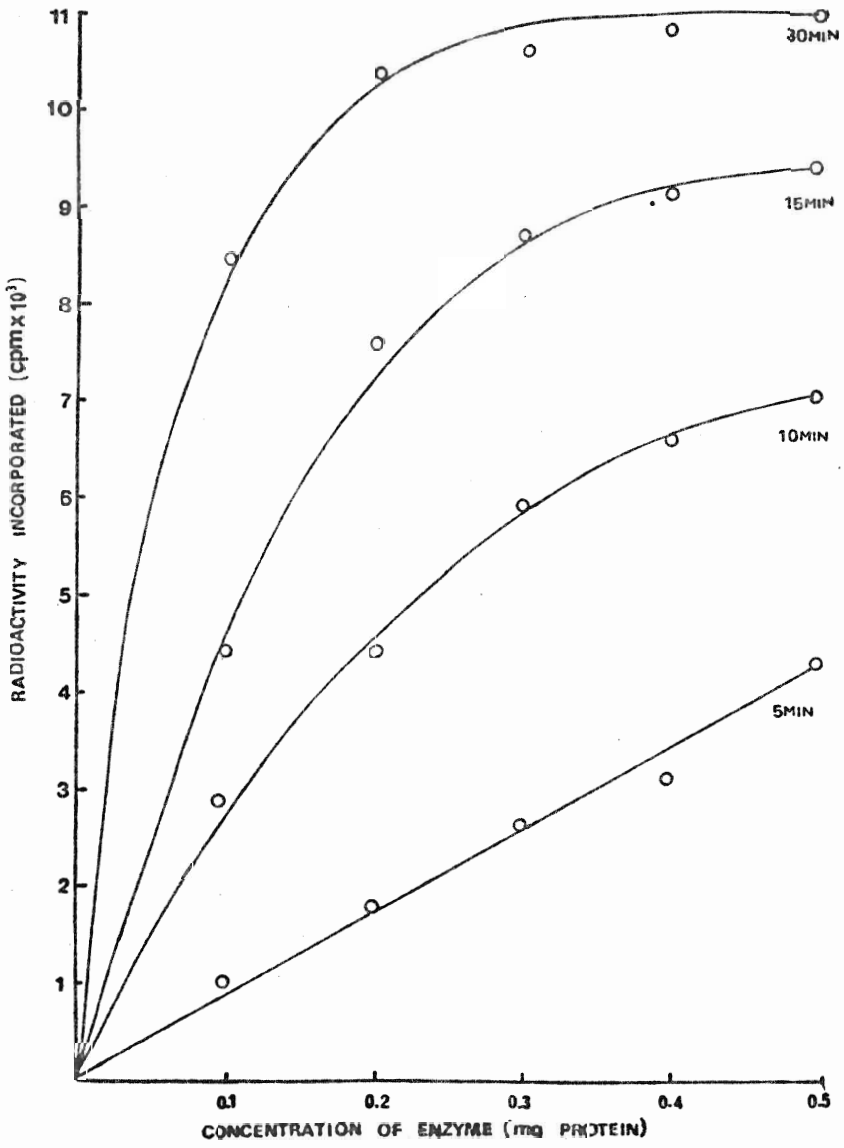
Appendix 3b. Effect of enzyme concentration on chitin synthesis in *M. pusilla*.

Conditions for assay were the same as described for M. candelabrum, except that the mixed membrane preparation was not activated with acid protease.

Appendix 3a.


RADIOACTIVITY INCORPORATED (cpmx10³)
10
9
8
7
6
5
4
3
2
1
0
0·1
0·2
0·3
0·4
0·5
CONCENTRATION OF ENZYME (mg PROTEIN)
30 MIN
15 MIN
10 MIN
5 MIN

Appendix 3b.


11
10
9
8
7
6
5
4
3
2
1
30MIN
15MIN
10MIN
5MIN
RADIOACTIVITY INCORPORATED (cpm x 10³)
0.1
0.2
0.3
0.4
0.5
CONCENTRATION OF ENZYME (mg PROTEIN)

Appendix 4. Calculation of enzyme activity

$$\text{Enzyme activity} = \frac{R_N}{2.22 \times 10^6 \times E/100 \times SA \times t} \times 10^{-3}$$

R_N	Net counting rate (cpm) of the radioactive product
E	Counting efficiency in percent
SA	Specific radioactivity ($\mu Ci\ \mu mole^{-1}$) of the substrate ($0.20\ \mu Ci\ \mu mole^{-1}$)
t	reaction time (min)
2.22×10^6	factor for conversion from disintegrations per min to μCi
10^{-3}	factor for conversion from $\mu mole$ to nmole of substrate

Appendix 5. Activation of chitin synthase by protease.

The experimental procedure was the same as described for Figure 11. Data show the ratio of chitin synthase activity with and without protease. The activation values were calculated from the highest response by the different proteases in the same enzyme preparation from Figure 11.

	Ratio of chitin synthase activity with and without protease		
Fungus	acid protease	neutral protease	trypsin
M. candelabrum	16.8	5.0	6.0
M. pusilla	2.1	1.2	1.5